Integrative Physiology

Acute Catecholamine Exposure Causes Reversible Myocyte Injury Without Cardiac Regeneration


Rationale: Catecholamines increase cardiac contractility, but exposure to high concentrations or prolonged exposures can cause cardiac injury. A recent study demonstrated that a single subcutaneous injection of isoproterenol (ISO; 200 mg/kg) in mice causes acute myocyte death (8%–10%) with complete cardiac repair within a month. Cardiac regeneration was via endogenous cKit+ cardiac stem cell–mediated new myocyte formation.

Objective: Our goal was to validate this simple injury/regeneration system and use it to study the biology of newly forming adult cardiac myocytes.

Methods and Results: C57BL/6 mice (n=173) were treated with single injections of vehicle, 200 or 300 mg/kg ISO, or 2 daily doses of 200 mg/kg ISO for 6 days. Echocardiography revealed transiently increased systolic function and unaltered diastolic function 1 day after single ISO injection. Single ISO injections also caused membrane injury in ≈10% of myocytes, but few of these myocytes appeared to be necrotic. Circulating troponin I levels after ISO were elevated, further documenting myocyte damage. However, myocyte apoptosis was not increased after ISO injury. Heart weight to body weight ratio and fibrosis were also not altered 28 days after ISO injection. Single- or multiple-dose ISO injury was not associated with an increase in the percentage of 5-ethyl-2-’-deoxyuridine–labeled myocytes. Furthermore, ISO injections did not increase new myocytes in cKit+Cre×R-GFP transgenic mice.

Conclusions: A single dose of ISO causes injury in ≈10% of the cardiomyocytes. However, most of these myocytes seem to recover and do not elicit cKit+ cardiac stem cell–derived myocyte regeneration. (Circ Res. 2016;119:865-879. DOI: 10.1161/CIRCRESAHA.116.308687.)

Key Words: catecholamine ■ drug ■ heart failure ■ myocardium ■ remodeling

Many forms of cardiovascular disease can lead to death of cardiac myocytes. Effective repair requires the generation of new cardiac muscle cells; however, the adult myocyte has lost all or most of its ability to reenter the cell cycle and divide. Therefore, stem cells with the capacity to differentiate into new cardiac myocytes could be useful for cardiac regeneration. Endogenous cKit+ cardiac stem cells (CSCs) were first described over a decade ago. There has been great debate over their ability to form new myocytes in the normal adult heart or to regenerate new cardiomyocytes after injury. Some studies suggest that these cells can robustly form new myocytes in the aging heart1 or in the adult heart after injury, whereas others find very small numbers of new myocytes during aging or after injury. A minority of studies suggest that these cells cannot differentiate into new myocytes.

Editorial, see p 779
In This Issue, see p 773

The rate of new myocyte formation (myocyte turnover) in the normal adult mammalian heart is also not well established, with recent studies reporting annual rates of myocyte turnover at 1%4 or 4% to 10%5 in humans and as low as 0.003%6 or as high as 8% to 10%7 in rodent studies using a variety of cell fate–mapping strategies.

The ability of the adult mammalian heart to regenerate in response to multiple forms of injury has been studied, and...
Experimental Animals
All animal procedures were approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. For most mouse experiments, wild-type 10- to 12-week-old male C57BL/6 mice (n=173) were used (The Jackson Laboratory, Bar Harbor, ME). For fate mapping of endogenous c-kit+ cardiac myocytes, constitutive and inducible Kit-Cre-IRESpGFP mice (n=9) or Kit-MerCreMer knock-in mice (n=5) were generated. In short, the Kit locus was targeted in mice to express Cre recombinase and eGFP (enhanced green fluorescent protein) with a nuclear localization sequence. These mice were crossed with Cre-responsive Rosa26 mice (R-GFP) for lineage tracing.

ISO Dosing
Single-dose ISO was administered by subcutaneous injections. We used 200 mg/kg ISO and a higher dose of 300 mg/kg (as this was the highest tolerable nonlethal dose). For the chronic ISO experiments, 200 mg/kg ISO was injected twice daily for 6 consecutive days. For all experiments, a stock solution of 50 mg/mL ISO (Sigma-Aldrich, No I6504, St Louis, MO) was prepared in sterile 0.9% saline.

Statistical Analysis
All data analyses were performed using SAS version 9.3. Data are shown as mean±SEM. A mixed-effects model approach was used for variables that were measured repeatedly. For variables that were measured only once, overall group comparisons were tested using the Kruskal–Wallis test or analysis of variance F test, when appropriate, whereas the pairwise group comparisons were performed using the exact Wilcoxon rank-sum test or unequal variance 2-sample t test.

Results
ISO Dosing
An ISO dose titration study was performed in mice to define a nonlethal ISO dose that injures the heart. Mice were injected with 5 (n=5), 200 (n=5), 300 (n=1), 400 (n=4), 500 (n=1), 600 (n=1), 700 (n=1), 800 (n=1), or 1000 mg/kg (n=5) ISO. Doses ≥400 mg/kg caused death of every animal within a few hours. Therefore, vehicle (saline), 200 mg/kg (dose used by Ellison et al10), and 300 mg/kg (highest nonlethal dose) were used for all experiments.

To document the lethal arrhythmias induced by high-dose ISO, continuous ECGs were recorded while animals were injected with 200, 300, 400, or 1000 mg/kg ISO. Injection of ISO induced many different types of arrhythmias. After injection of 400 and 1000 mg/kg, ECG tracings initially revealed a slight increase in heart rate (data not shown) followed by sinus arrest and heart block with a ventricular escape rhythm within a few minutes that ended either in lethal ventricular tachycardia or asystole (Online Figure I).

ISO Transiently Increases Myocardial Contractility and Does Not Depress Diastolic Function
Mice were injected subcutaneously with vehicle (n=14), 200 mg/kg (n=15), or 300 mg/kg (n=14) ISO. All animals underwent baseline echocardiography (ECHO) before dosing and then at 1, 3, 7, 14, and 28 days after ISO/vehicle injection. Conventional ECHO revealed that 200 and 300 mg/kg ISO significantly increased left ventricular (LV) ejection fraction (EF) and fractional shortening (FS) after 1 day compared with baseline. Injection with 300 mg/kg ISO significantly increased EF (59.9±0.9% versus 69.3±2.5%; P<0.01) and FS (31.2±0.6% versus 38.8±2.1%; P<0.01) after 1 day compared with the vehicle-injected group (Figure 1A and 1B; Online Movies I–IV).

Methods
Please refer to the Materials and Methods section in the Online Data Supplement for a detailed description of experimental methods.
Cardiac function returned to baseline levels at 1 week and was unchanged for the remainder of the study. End-systolic and end-diastolic volumes did not differ between groups at any time point (Figure 1C and 1D). Figure 1E shows representative M-mode images of all 3 treatment groups at baseline and 1 day post ISO injection. Heart rates during ECHO did not significantly differ between groups at any time point (Online Figure I).

Diastolic function was determined using ECHO coupled with pulsed Doppler and tissue Doppler imaging techniques. No differences in diastolic function ($E$, $E/A$, $E'$, $EAT$ [acceleration time of the transmitral early diastolic peak flow velocity]; Online Figure II) between groups at any time point were found.

Subendocardial and Apical Myocardial Contractility Are Not Impaired After ISO Injury

To determine the effects of ISO on regional myocardial function, speckle tracking–based strain analyses on long-axis B-mode loops were performed. Because of myocardial fiber orientation at varying levels of the LV wall, longitudinal strain is most representative of myocardial shortening at the level of the endocardium. Furthermore, the software allows analysis of regional wall segments. Consistent with the results from conventional ECHO, 300 mg/kg ISO significantly increased the magnitude of global longitudinal strain (Figure 2A) compared with vehicle controls after 1 day ($-22.26\pm0.98\%$ versus...
−18.48%±0.64%; \( P<0.01 \)) and returned to baseline after 3 days. Apical longitudinal strain (Figure 2B) was not different between the groups. Strain rate reflects the rate of changes in strain (strain/time). Both global and apical strain rates (Figure 2C and 2D) were significantly improved by 300 mg/kg ISO after 1 day compared with baseline. In line with our previous ECHO findings, this effect was transient. After 3 days, global and apical longitudinal strain, as well as strain rate, returned to baseline values and remained unchanged throughout the study. Figure 2E and 2F show normal 3-dimensional
Figure 3. Isoproterenol (ISO) induced myocyte membrane damage and does not induce apoptosis. A–C, Tissue sections from hearts fixed at 1 day post injection immunostained for terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end-labeling (TUNEL; green), tropomyosin (red), and nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, blue). Vehicle (A), 200 mg/kg ISO (B), and 300 mg/kg ISO (C). White arrowheads indicate myocyte nuclei. Scale bars=25 μm. D–F, Hematoxylin and eosin (Continued)
regional wall velocity diagrams and vector diagrams of all 3 groups, respectively, 1 day after injection. These results demonstrate that a single subcutaneous ISO injection (200 or 300 mg/kg) does not impair subendocardial or apical contractility and, therefore, from a functional point of view, does not produce a takotsubo-like cardiomyopathy in these mice.

Single ISO Injection Alters Myocyte Membrane Permeability and Induces Necrosis but Not Apoptosis In Vivo

Comprehensive functional assessment of myocardial function revealed no functional deterioration after ISO injection at any time point. Injection with 300 mg/kg ISO even increased systolic function transiently. We next explored the idea that ISO induces cardiac myocyte injury. To investigate the effects of ISO on myocyte and nonmyocyte apoptosis, we performed terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining on myocardial tissue fixed at 1 day after ISO injection. Figure 3A–3C displays representative confocal micrographs of all 3 groups 1 day after injections, with the magnified panels showing TUNEL+ nuclei. There were no significant increases in the percentage of TUNEL+ myocytes after ISO treatment compared with vehicle (Figure 3H). The percentage of TUNEL+ nonmyocytes was slightly increased after ISO, but this did not reach statistical significance (Figure 3I). Confirming these results, Western blot analysis revealed no increase in activated caspase-3 after ISO injection (Online Figure III).

To further investigate ISO-induced myocyte injury, myocyte membrane permeability and necrosis were evaluated by measuring Evans Blue Dye (EBD) uptake 24 hours after ISO or vehicle injection. Figure 3D–3F shows representative images of EBD uptake and hematoxylin and eosin (H&E) staining of the matched histological sections from all 3 groups. Quantification of myocyte EBD uptake (Figure 3G) revealed a significant increase after 200 mg/kg (9.79±0.58%) and 300 mg/kg ISO (8.04±0.58%) compared with vehicle (0.04±0.02%; P<0.05). EBD uptake certainly defines ISO-induced membrane damage but does not necessarily document myocyte necrosis.17,18 We found areas of EBD+ myocytes where the matching H&E-stained section revealed intact, nonnecrotic myocytes (Figure 3E and 3F, lower small images). We also identified EBD+ myocytes with histological features of necrosis (Figure 3E and 3F, upper small images). These results suggest that ISO can alter myocyte membrane permeability without necessarily causing necrosis. EBD+ myocytes were found throughout the ventricle and were not localized exclusively to the endocardium or the apical regions of the LV.

To further characterize injury induced by ISO, troponin I (Tn-I) levels were measured 24 hours after ISO injection in vehicle, 200 mg/kg, and 300 mg/kg ISO-injected mice and after MI induced by permanent occlusion of the left anterior descending artery, as a positive control (Figure 3J). Tn-I levels were significantly increased after 300 mg/kg ISO (8.2±1.9 ng/mL; P<0.05) and after MI (58.1±7.1 ng/mL; P<0.01) compared with vehicle, whereas 200 mg/kg ISO caused only a slight increase in Tn-I, which did not reach statistical significance (0.4±0.2 ng/mL; P=NS). Injury with 300 mg/kg ISO caused a significantly higher increase in Tn-I levels compared with 200 mg/kg (P<0.05). Notably, compared with Tn-I levels after MI, 200 and 300 mg/kg ISO caused significantly smaller increases (P<0.01). In the vehicle group, the Tn-I levels were too low for quantification (<0.01 ng/mL). These results suggest that a single injection of either 200 or 300 mg/kg ISO caused transient changes in myocyte membrane permeability but that not all EBD+ myocytes were necrotic.

Single ISO Injection Does Not Induce Cardiac Fibrosis or Change Heart Size in C57BL/6 Mice

Myocardial injury from MI,15 pressure overload, or prolonged ISO infusion16 elicits a fibrotic response that is associated with changes in myocardial diastolic compliance. If ISO caused significant myocardial necrosis, an increase in fibrotic area would be expected. Because we observed a significant increase in EBD uptake after ISO treatment, which is associated with myocyte injury,19 we measured cardiac fibrosis. Masson’s trichrome–stained tissue sections from hearts fixed at 4 weeks post ISO or vehicle injections were studied (Figure 4A–4C shows representative images). Bioquantification analysis did not show significant differences in the percentage of fibrotic tissue between groups (Figure 4G). Furthermore, no changes in heart weight to body weight ratio (HW/BW) at 4 weeks post ISO were observed (Figure 4H), documenting the lack of organ hypertrophy.

Diseases that induce cardiac myocyte death are usually associated with hypertrophy of surviving myocytes. To explore this possibility, myocyte cross-sectional area was measured in Masson’s trichome–stained tissue sections (Figure 4D–4F and 4I), and cell surface area was measured in myocytes isolated from vehicle and ISO-injected animals (Figure 6B). A trend toward an increase in myocyte size after ISO injury was observed, but this effect did not reach statistical significance. To further explore the idea that ISO injury induces reactive pathological hypertrophy, mRNA expression levels of the hypertrophic markers atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain were measured at 1 and 4 weeks post ISO. In the 300 mg/kg ISO-treated animals compared with vehicle-treated controls (Figure 4J), significant increases in atrial natriuretic peptide and β-myosin heavy chain expression patterns were observed at 1 week. After 200 mg/kg ISO injection, β-myosin heavy chain was also upregulated at 1 week. β-Myosin heavy chain expression in both groups was found throughout the ventricle and were not localized exclusively to the endocardium or the apical regions of the LV.
Figure 4. Myocardial fibrosis and hypertrophy. A–C, Tissue sections from hearts fixed at 4 weeks post isoproterenol (ISO) injection (vehicle (A and D); 200 mg/kg ISO (B and E), and 300 mg/kg ISO (C and F)) were stained with Masson’s trichrome. Bright-field micrographs with bioquantification indicating Masson’s trichrome blue–stained fibrotic areas (green). Scale bars=150 μm. D–F, Representative bright-field micrographs of vehicle (D), 200 mg/kg ISO (E), and 300 mg/kg ISO (F) hearts studied 4 weeks after injection. Scale bars=50 μm. G, Percentage of fibrotic area determined by bioquantification (n=9, 3 each group). H, Heart weight/body weight ratio (HW/BW; n=14). I, Average myocyte cross-sectional area (n=9, 3 each group). J, Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (βMHC). mRNA levels at 1 week (n=11) and 4 weeks (n=12) after vehicle, 200 mg/kg ISO, and 300 mg/kg ISO. ANP and βMHC expression levels were upregulated at 1 week post ISO (300 mg/kg). *P<0.05 vs veh 1 wk, #P<0.05 vs veh 4 wk, γP<0.05 1 wk vs 4 wk, NS=not significant (P>0.05). G–I, Data are shown as mean±SEM. CM indicates cardiomyocytes.
(200 and 300 mg/kg ISO) had returned to normal by 4 weeks post ISO injection. Collectively, our results suggest that a single ISO injection causes a small amount of cardiac myocyte necrosis and a small amount of reactive cardiac hypertrophy, with no detectable fibrosis.

ISO Does Not Increase EdU Incorporation in Myocyte Nuclei In Vivo

Our experiments show that ISO caused myocyte injury (EBD uptake in 10% of ventricular myocytes) but was not associated with an increase in fibrosis. These findings could be because of necrotic myocytes being replaced by CSC-derived new myocytes as suggested in the previous study10 or because most of the damaged and EBD-labeled myocytes recover normal function. To assess whether ISO injury induces increased myocyte and nonmyocyte proliferation and DNA replication in vivo, an EdU pulse-chase labeling strategy was used. A similar approach with 5-bromo-2′-deoxyuridine (BrdU) has been used previously by our group to identify newly generated feline myocytes after cardiac injury caused by 10 days of continuous ISO infusion.5 Figure 5A–5C displays representative confocal micrograph images of hearts treated with a single injection of vehicle or ISO (200 and 300 mg/kg) 1 week after injection and EdU labeling (via minipump). Quantitative histology (Figure 5D) revealed no differences in the percentage of EdU+ myocytes between ISO- and vehicle-treated animals at 4 weeks (vehicle 0.08±0.01%, 200 mg/kg ISO, 300 mg/kg ISO 0.09±0.03%; P=NS). ISO caused a significant dose-dependent increase in the percentage of EdU+ nonmyocytes (vehicle 0.47±0.12%, 200 mg/kg ISO 2.5±0.15%, and

Figure 6. Isoproterenol (ISO) did not increase the percentage of EdU+ myocyte nuclei in isolated cardiomyocytes. A, The percentage of EdU+ cardiomyocytes (CM) was <0.008% and did not differ between groups (veh [n=3], 200 mg/kg ISO [n=3], and 300 mg/kg ISO [n=4]) at 4 weeks post ISO. B, Cardiomyocyte surface area at 4 weeks post ISO. C, Representative fluorescent micrograph of a small EdU+ mononucleated myocyte. Scale bars=50 μm. Green arrowheads indicate cardiomyocyte. White arrowheads indicate myocyte nuclei. D, The percentage of mono-, bi-, and multinucleated cells in all CMs (left) and EdU+ CMs (right) at 4 weeks post ISO. NS indicates not significant (P>0.05). Data are shown as means±SEM. EdU indicates 5-ethynyl-2′-deoxyuridine.
300 mg/kg ISO 3.57±0.53%; P<0.05; Figure 5E). After MI, a much larger number of EdU⁺ nonmyocyte nuclei were observed in the injured border zone and infarct area, with fewer in the viable tissue of the remote zone (Online Figure IV). These results suggest that ISO causes a dose-dependent injury that elicits a proportional postinjury inflammatory response.

To more conclusively document the percentage of EdU⁺ myocytes, single cells were isolated from vehicle, 200 mg/kg, or 300 mg/kg ISO-treated mice 4 weeks post ISO injection (Figure 6). The percentage of EdU⁺ cardiomyocytes was low and did not differ between groups (vehicle 0.007±0.003%, 200 mg/kg 0.006±0.003%, and 300 mg/kg 0.008±0.001; P=NS). Many of the EdU⁺ myocytes were small and mononucleated (Figure 6C). Sixty-three percent of EdU⁺-isolated cardiomyocytes were mononucleated, 34% binucleated, and 3% multinucleated, whereas among all myocytes, 9.4% were mononucleated and 84.5% were binucleated (Figure 6D). These results suggest that there is a low rate of new myocyte formation in the adult C57BL/6 mouse, and this rate is not significantly increased after cardiac injury via a single injection of ISO.

ISO Does Not Increase CSC-Derived New Myocytes

To determine whether the new myocytes seen in these studies were derived from CSCs, transgenic mice that use the Kit locus for lineage tracing were used.6 The constitutive Kit⁺Cre⁺R-GFP mice (Kit⁺Cre⁺R-GFP) irreversibly mark any cell that previously or currently expresses cKit with green fluorescent protein (GFP).6 These mice were injected with 200 mg/kg ISO, and after 4 weeks, myocytes were isolated or hearts were fixed for immunostaining. Figure 7A displays a representative fluorescent micrograph of an eGFP⁺ myocyte. Quantification of eGFP⁺ fluorescent myocytes (Figure 7D) from the hearts of ISO-injected Kit⁺cre⁺R-GFP mice revealed a similar percentage of eGFP⁺ myocytes compared with untreated Kit⁺cre⁺R-GFP mice (0.0055±0.0004% versus 0.0053±0.0007%; P=NS). The low percentage of eGFP⁺ myocytes is similar to that reported previously by van Berlo et al.6

Tissue sections from hearts fixed at 4 weeks were stained for GFP (green) and α-sarcomeric actin (red; Figure 7E). Sixteen sections from each heart were analyzed for GFP⁺ myocytes. One to two GFP⁺ myocytes were identified per entire heart section, which is consistent with findings from van Berlo et al.6 The majority of GFP⁺ cells in every section appeared to be endothelial cells. Furthermore, none of the GFP⁺ myocytes were positive for EdU (Figure 7F). The EF in Kit⁺cre⁺R-GFP mice and nontransgenic littermates was not impaired after 200 mg/kg ISO injection at any time point (Online Figure V). The functional properties of eGFP⁺ myocytes were also studied. FS (Figure 7B and 7G) and Ca⁺²-transients (Figure 7C and 7G) did not differ between GFP⁺ and GFP⁻ myocytes isolated from the same hearts. Thus, a single injection of 200 mg/kg ISO does not increase the percentage of cKit⁺-derived new myocytes in the Kit⁺cre⁺R-GFP mice or alter the physiology of myocytes isolated from them.

Chronic ISO Causes Cardiac Injury Without Relevant New Cardiomyocyte Formation

We were concerned that our failure to see myocyte regeneration after a single ISO injection might have resulted from insufficient myocyte injury. Therefore, C57BL/6 mice were injected with 200 mg/kg ISO twice daily for 6 consecutive days. In contrast to a one-time ISO injection, multiple ISO injections caused a significant decrease in EF at 7 and 28 days post injection compared with vehicle-injected animals (28 days: ISO 48.0±2.9% versus vehicle 56.6±1.1%; P<0.05; Figure 8A; Online Figure VI). Furthermore, diastolic function, represented by an increased E/A ratio, was also significantly impaired at 7 and 28 days after ISO injection (28 days: ISO 3.8±0.5 versus vehicle 1.5±0.04; P<0.01; Figure 8B; Online Figure VI). However, the percentages of fibrotic area (data not shown), myocyte cross-sectional area (Online Figure VI), and HW/BW ratio (Online Figure VI) were comparable to those of vehicle-injected mice, consistent with our findings after single ISO injection. The EdU pulse-chase labeling strategy revealed no differences in the percentage of EdU⁺ myocyte nuclei between ISO- and vehicle-treated animals at 28 days after injections (ISO 0.12±0.03% versus vehicle 0.11±0.01%; P=NS; Figure 8C). The percentage of EdU⁺ nonmyocyte nuclei was significantly increased after multiple ISO injections (ISO 6.4±0.71% versus vehicle 0.5±0.06%; P<0.01; Figure 8D). This significantly exceeded the percentage of EdU⁺ nonmyocyte nuclei after both 200 and 300 mg/kg single ISO injections (fold-change: 200 mg/kg 5±0.31, 300 mg/kg 7.1±1.07, and chronic ISO 12.8±1.43; P<0.05; Online Figure VI).

To further explore if a more pronounced cardiac injury elicited CSC-mediated new cardiomyocytes, Kit⁺MCM⁺R-GFP mice were injected with 200 mg/kg ISO twice daily for 6 consecutive days. Before ISO dosing, Kit⁺MCM⁺R-GFP mice were induced with tamoxifen for 2 weeks to activate the lineage tracing system.6 In line with our findings in C57BL/6 mice, myocyte cross-sectional area (Online Figure VI), HW/BW ratio (Online Figure VI), and the percentage of EdU⁺ myocyte nuclei (ISO 0.04±0.01% versus vehicle 0.08±0.02%; P=NS; Figure 8H) were unaltered after ISO. Although there was a 4-fold increase in the percentage of EdU⁺ nonmyocyte nuclei (ISO 15.6±1.64% versus vehicle 3.8±0.45%; P=NS; Figure 8I), as well as an increase in the percentage of fibrotic area (ISO 2.7±0.13% versus vehicle 1.6±0.13% versus vehicle 0.9±0.01%; P=NS; Figure 8G; Online Figure VI) in the ISO group compared with vehicle, both did not reach statistical significance, which is most likely explained by a small sample size. However, we did not observe any increase in eGFP⁺ myocytes. Thus, chronic ISO exposure caused more robust cardiac injury and depressed systolic and diastolic function, but did not elicit ckit⁻-CSC-derived myocyte regeneration.

Discussion

The objective of this study was to validate published work10 demonstrating that a single injection of ISO in mice induces myocyte necrosis localized to the ventricular endocardium and apex, with transient depression of cardiac pump function, followed by a regenerative response in which CSCs are activated to proliferate and differentiate into new cardiac myocytes that fully regenerate the myocardium. Our goal was to use this system to study the properties of newly forming adult cardiac myocytes. We conducted a dose titration study in C57BL/6 mice and demonstrated that ISO doses >400 mg/kg were lethal. We then explored the effects of single injections of 200 mg/kg (the concentration used in the previous
study\(^{19}\), the highest nonlethal dose of 300 mg/kg, and chronic ISO administration for 6 days (to increase injury). We defined the effects of these ISO doses on cardiac systolic and diastolic function, myocyte injury, myocyte size, cardiac fibrosis, and new myocyte generation.

**Figure 7.** Isoproterenol (ISO) injury did not increase the percentages of ckit\(^+\) CSC-derived new myocytes or alter their physiology. A, Confocal micrographs of an eGFP\(^+\) myocyte isolated from a transgenic kit\(^{+/cre}\)×R-GFP mouse. Average fractional shortening (FS) data (B), peak Ca\(^{2+}\)-transient measurements (C), FS traces and Ca\(^{2+}\) transients (G) in eGFP\(^+\) myocytes vs normal non-GFP\(^+\) (GFP\(^-\)) myocytes from the same heart. D, 200 mg/kg ISO injection did not alter the percentage of eGFP\(^+\) myocyte compared with untreated kit\(^{+/cre}\)×R-GFP mouse. E and F, Representative confocal micrographs of fixed hearts from transgenic kit\(^{+/cre}\)×R-GFP mice at 4 weeks after ISO (200 mg/kg) injection that were immunostained against GFP (green), α-sarcomeric actin (α-SA; red in E; gray in F), 5-ethynyl-2′-deoxyuridine (EdU; red), and nuclei labeled with 4′,6-diamidino-2-phenylindole (DAPI, blue). White arrowheads indicate Edu\(^+\) nuclei. BF indicates bright field. Scale bars=50 μm. NS=not significant (P<0.05). B–D, Data are shown as mean±SEM. See also Online Figure V.

**Single Injection of High-Dose ISO Causes Transient-Positive Inotropic Effects**

ISO is a potent β-adrenergic agonist that increases systolic [Ca\(^{2+}\)] to enhance myocyte contractility.\(^{20}\) Toxic levels of catecholamines or prolonged exposure can induce cardiac injury.
Figure 8. Chronic isoproterenol (ISO) administration caused cardiac injury without new myocyte formation. A–F, C57BL/6 mice were injected with 200 mg/kg ISO (n=9) or vehicle (n=5) twice daily for 6 days. A and B, ISO caused significant systolic (ejection fraction) and diastolic (E/A) impairment in the ISO group (n=9) compared with vehicle group (n=5). C and D, The percentage of EdU+ myocyte (C) or nonmyocyte (D) nuclei (veh [n=4], ISO$_{chron}$ [n=5]). E and F, Confocal micrographs of fixed hearts at 4 weeks post injection: veh (E) and ISO$_{chron}$ (F) that were immunostained against α-sarcomeric actin (α-SA, red), 5-ethyl-2′-deoxyuridine (EdU, green), and nuclei (Continued)
in part by inducing Ca\(^{2+}\) overload–mediated myocyte necrosis.\(^{21}\)

The previous study\(^{10}\) showed that a single injection of 200 mg/kg ISO caused endocardial and apical myocyte death and reduced cardiac function. To validate these observations, we first performed comprehensive ECHO evaluation for 4 weeks after ISO injection, and we further performed speckle tracking–based strain imaging, a high-sensitivity method for phenotyping cardiac function. These techniques are known to detect small global and regional changes in contractility.\(^{16,22}\) Neither conventional nor strain analyses revealed any functional deterioration at any time point after ISO injection. In fact, we observed a transient enhancement of myocardial contractility (EF, FS, global longitudinal strain; Figures 1A, 1B, and 2A) 1 day after ISO injection compared with vehicle, consistent with the well-known positive inotropic action of ISO. Ellison et al\(^{10}\) did not present ECHO data in mice, only hemodynamic measurements, where they demonstrated a significant decrease in LV-developed pressure (+dP/dt) 1 day post ISO exposure. These data suggest that apoptosis plays a minor role in ISO-induced myocardial injury, which is consistent with Ellison et al’s\(^{10}\) observations in mice and rats. We found that serial subcutaneous ISO administration induces hypertrophy, diastolic dysfunction, and increased myocardial fibrosis, but does not affect systolic function.\(^{26,27}\) Kudej et al demonstrated that chronic ISO administration via minipumps induced cardiac hypertrophy without cellular necrosis.\(^{31}\) Hohimer et al highlighted that different routes of ISO administration have different hemodynamic sequelae and could potentially evoke different cardiac phenotypes in mice.\(^{32}\) In our view, the variability in ISO effects on cardiac function reported in the literature likely results from different ISO dosing strategies.

### ISO Causes Myocyte Injury Without Inducing Apoptosis or Widespread Necrosis

We did not observe a significant increase in the percentage of TUNEL\(^{+}\) myocytes (Figure 3H) or nonmyocytes (Figure 3I) or an increase in activated caspase-3 (Online Figure III) after ISO exposure. These data suggest that apoptosis plays a minor role in ISO-induced myocardial injury, which is consistent with Ellison et al’s\(^{10}\) observations in mice and rats. We found that 200 and 300 mg/kg ISO caused ≈10% of the ventricular myocytes to take up EBD, documenting membrane permeability abnormalities.\(^{19}\) EBD uptake is further associated with loss of membrane integrity and loss of mitochondrial membrane potential.\(^{13,14}\) Myocardial necrosis after ISO administration has been reported in several animal models,\(^{3,5,10,23,24,34,35}\) but we did not find global functional defects or cardiac fibrosis. Therefore, we questioned if EBD\(^{+}\) myocytes were indeed all destined for necrosis. H&E-stained slides of the matched EBD\(^{+}\) sections showed some areas in which EBD\(^{+}\) myocytes showed features of necrotic tissue (Figure 3E and 3F, upper small images). However, many areas with EBD\(^{+}\) myocytes showed no other histological features of necrosis (in the matched H&E sections; Figure 3E and 3F, lower small images). Others have shown that ISO can induce a transient alteration in sarcolemmal membrane permeability without necrosis.\(^{37}\) A transient membrane instability could cause EBD uptake in some myocytes, with subsequent recovery after elimination of the stressor. Although Ellison et al\(^{10}\) reported that 200 mg/kg ISO caused the same type of myocardial damage in mice and rats, they did not document how they measured myocardial
necrosis in mice. Interestingly, they reported that 200 mg/kg ISO caused necrosis in 10% of myocytes, which is similar to our findings with EBD uptake. In our study, Tn-I levels measured 1 day after ISO injection were significantly elevated after 300 mg/kg ISO, but not after 200 mg/kg ISO. In addition, we showed that ISO-injured animals had significantly lower levels of serum Tn-I levels compared with mouse hearts with MI (which clearly induces myocyte death, inflammation, and reactive fibrosis); Figure 3J). In humans, Tn-I levels correlate with myocardial necrosis after radiofrequency ablation and infarct volume after acute MI. A close correlation between cardiac troponin T and histological infarct size has been observed in mice. Interestingly, the increase in Tn-I after 300 mg/kg ISO was significantly higher compared with 200 mg/kg ISO, which suggests increased injury. The percentage of EBD+ myocytes was similar between 200 and 300 mg/kg ISO, suggesting that EBD uptake may identify both damaged (but able to recover) and necrotic cells. Collectively, our results suggest that a single sublethal ISO injection in C57BL/6 mice causes transient membrane damage in >10% of the myocytes, and some of these myocytes seem to undergo necrosis with a small, ISO dose-dependent increase in the infiltration of proliferative inflammatory cells, but no reactive fibrosis.

**ISO Causes Dose-Dependent Injury Without Inducing Cardiac Regeneration**

We used an EdU pulse-chase labeling strategy to identify newly forming myocytes and nonmyocytes at 4 weeks after ISO injury. Newly formed myocytes (from any source) that entered the cell cycle would be detected by EdU nuclear incorporation. The study by Ellison et al showed that 8% to 10% of the ventricular myocytes were labeled with BrdU 6 and 28 days after 200 mg/kg ISO. We did not find any increase in EdU+ myocytes at 4 weeks after injection with 200 or 300 mg/kg ISO (Figure 5D). We used EdU rather than BrdU in our study because, in our hands, detection of EdU is much more reliable. The smaller molecular size of the EdU reagents, higher diffusion rates, and more effective tissue penetration without denaturation allows for better structural preservation. EdU could also label damaged DNA; thus, it is possible that we detected false-positive proliferating cells. Given the fact that only a few cardiomyocytes were EdU+ (<0.10%) and the fact that most of these myocytes were small and mononucleated makes the idea that they represent damaged DNA rather than new DNA highly unlikely. Our findings suggest that there is a low rate of new myocyte formation in the adult mouse heart, and the rate of new myocyte formation is not increased significantly after ISO injury. The idea that the EdU+ myocytes represent newly formed myocytes was strongly supported by our finding that these cells were largely mononucleated, whereas the majority of EdU+ myocytes were binucleated. We also explored whether ISO injury induces cardiac myocyte regeneration and whether new myocytes are derived from CSCs. We used a cKit lineage fate-mapping strategy in which Kit+SCs (BrdU labeling). However, 28 days after ISO injury, BrdU labeling of ventricular myocytes increased by only 0.04%, suggesting that larger amounts of ISO injury than EdU+-isolated myocytes observed after ISO injury or in controls was similar to the percentage of GFP+ myocytes derived from CSCs (≈0.005%). The somewhat higher percentage of EdU+ myocytes found in tissue sections could simply reflect differences in experimental approaches, such as difficulties with reliable identification of myocyte nuclei in tissue sections. Interestingly, none of the GFP+ myocytes we identified after ISO injury had an EdU+ nucleus (Figure 7F). These findings suggest that the new EdU+ myocytes we observed may not have been derived from a cKit-expressing precursor cell.

**Chronic ISO Causes Cardiac Injury Without Activating CSC-Mediated Regeneration**

Multiple high-dose ISO injections were used to determine if a more pronounced cardiac injury could induce cardiomyocyte regeneration. In C57BL/6 mice, multiple high-dose ISO injections caused significant impairments in systolic and diastolic function (Figure 8A and 8B; Online Figure VI) and increased the percentage of EdU+ nonmyocyte nuclei (Figure 8D; Online Figure VI), but not of EdU+ myocytes (Figure 8C). Interestingly, we could not find evidence for significant reactive hypertrophy (normal HW/BW and myocyte cross-sectional area) after multiple ISO injections in C57BL/6 mice. In line with the findings in C57BL/6 mice, multiple high-dose ISO injections increased the percentage of EdU+ nonmyocyte nuclei (Figure 8I) in Kit+MCM+R-GFP mice. Myocyte cross-sectional area (Online Figure VI), HW/BW ratio (Online Figure VI), and the percentage of EdU+ myocyte nuclei (Figure 8H) were also not altered after multiple high-dose ISO injections in Kit+MCM+R-GFP mice. Interestingly, ISO caused LV fibrosis in Kit+MCM+R-GFP (Figure 8G; Online Figure VI) but not in C57BL/6 mice (data not shown). The difference might be related to tamoxifen induction before ISO dosing in Kit+MCM+R-GFP mice. Furthermore, we cannot rule out strain- and sex-related differences in the response to ISO. Faulx et al reported that ISO causes a more pronounced cardiac injury in A/J than C57BL/6 mice, because of β-adrenergic receptor (β-AR) downregulation in C57BL/6 mice. However, we do not think that sex and strain differences can account for the differences between Ellison’s and our findings.

The percentage of EdU+ myocytes did not increase after multiple high-dose ISO administration, both in C57BL/6 and in Kit+MCM+R-GFP mice, suggesting that the rate of new myocyte formation is not significantly increased after this form of ISO injury. Notably, we did not observe an increase in eGFP+ myocytes either after multiple ISO injections (Kit+MCM+R-GFP mice), which is in line with our findings in Kit+CSCs (R-GFP mice) after a single 200 mg/kg ISO injection. Our findings suggest that ISO induces dose-dependent cardiac injury (Tn-I levels, EdU+ nonmyocyte nuclei), but despite induction of several pathological alterations, neither single nor multiple ISO injections induced cardiomyocyte regeneration. In a previous study in felines, continuous ISO infusion for 10 days via osmotic minipumps was shown to induce myocyte death, cardiac fibrosis, and depressed cardiac contractile function. This strategy for ISO injury was shown to activate cKit+ CSC (BrdU labeling). However, 28 days after ISO injury, BrdU labeling of ventricular myocytes increased by only 0.04%, suggesting that larger amounts of ISO injury than
caused in the present study induces a small regenerative response. The source of these new myocytes in the feline study could not be determined conclusively.

In summary, the current study shows that a single injection of 200 or 300 mg/kg ISO caused myocyte membrane permeability alterations sufficient to allow EBD uptake and Th-1 leak from ~10% of the ventricular myocytes. These injured myocytes were observed diffusely throughout the heart and were not localized to the endocardium or apical regions of the heart. No reductions in global or regional myocardial performance were observed. Therefore, a single dose of 200 mg/kg ISO in mice does not produce a takotsubo-like cardiomyopathy in C57BL/6 mice. A small amount of myocyte necrosis was induced by ISO injury, and we found a small, ISO dose–dependent increase in the infiltration of proliferative inflammatory cells but no significant myocardial fibrosis that is normally associated with widespread necrotic injury. Multiple ISO injections caused a more pronounced cardiac injury with systolic and diastolic cardiac dysfunction. Although ISO induced a dose-dependent cardiac injury, we did not find any evidence for cardiac regeneration either after a single or after multiple injections of high-dose ISO, using 2 independent techniques and 2 ckit lineage tracing mouse models. Collectively, our results in mice do not support the idea that ISO injury elicits cardiomyocyte regeneration.

Acknowledgments

We thank Sarah Anne Troupch for her assistance in editing the article.

Sources of Funding

Supported by National Institutes of Health (NIH) grants R01HL089312, T32HL091804, P01HL097199, and R01HL033921 to S.R. Houser. S. Mohsin received an SDG grant from American Heart Association.

Disclosures

None.

References


A previous report showed that a single injection of isoproterenol (ISO; 100 mg/kg) causes myocardial injury in a cKit+ lineage tracing mouse model. ISO injury in a cKit+ lineage tracing mouse model does not result in cardiomyocyte regeneration. Single or multiple injections of ISO cause injury but do not induce an increase in the number of newly generated cardiomyocytes, as determined by ethynyl deoxyuridine labeling. ISO injury in a cKit+ lineage tracing mouse model does not result in new myocyte generation from cKit+ precursor cells.

What New Information Does This Article Contribute?

- A single ISO injection (200 or 300 mg/kg) in C57BL/6 mice causes dose-dependent injury in 8% to 10% of the ventricular myocytes, but hearts recover without significant fibrosis or abnormal systolic and diastolic cardiac function.
- Single or multiple injections of ISO cause injury but do not induce an increase in the number of newly generated cardiomyocytes, as determined by ethynyl deoxyuridine labeling.
- ISO injury in a cKit+ lineage tracing mouse model does not result in new myocyte generation from cKit+ precursor cells.

The adult mammalian heart seems to have a limited ability to regenerate myocytes that die with disease or aging. Developing novel approaches to replace lost myocytes and their supportive vasculature would be a major medical advance. Endogenous cKit+ cardiac stem cells are thought to have the capacity to differentiate into new myocytes and vascular tissue. We addressed the question of whether cardiac stem cells can significantly regenerate the adult heart after ISO-induced injury. ISO caused significant myocyte injury and infiltration of proliferative inflammatory cells. However, after recovery, no significant late myocardial fibrosis or impairment of systolic and diastolic cardiac function was found. Although ISO induced a dose-dependent increase in cardiac injury, the percentage of EdU+ cardiomyocytes did not increase.

What Is Known?

- Endogenous cKit+ cardiac stem cells have the ability to form new myocytes in the normal adult heart and to regenerate new cardiomyocytes after cardiac injury. However, the extent to which cardiac stem cells form new myocytes after cardiac injury is highly variable in different studies.
- High levels of circulating catecholamines can induce reversible cardiac stunning and myocyte death.
- A previous report showed that a single injection of isoproterenol (ISO; 200 mg/kg) kills 8% to 10% of the myocytes in the mouse heart, and over the 28 days, these lost myocytes are regenerated from cKit+ cardiac stem cells.

Novelty and Significance
Acute Catecholamine Exposure Causes Reversible Myocyte Injury Without Cardiac Regeneration


Circ Res. 2016;119:865-879; originally published online July 26, 2016; doi: 10.1161/CIRCRESAHA.116.308687

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/119/7/865

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/07/26/CIRCRESAHA.116.308687.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Detailed Methods

Animals
All animal procedures were approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. Anesthesia was induced and maintained using inhaled isoflurane (Butler Shein Animal Health; Dublin Ohio). For most mouse experiments, wild-type 10-12-week-old male C57BL/6 mice were used (The Jackson Laboratory; Bar Harbor, ME). For fate mapping of endogenous c-kit+ cardiac myocytes, constitutive and inducible Kit-Cre-IRESGFPnls or Kit-MerCreMer knock-in mice were generated as described previously via aggregation of gene-targeted ES cells with 8-cell embryos to generate chimeric mice on a mixed B6.129S4 background. Animals were then backcrossed to C57Bl/6J (Jackson Labs #000664) for a minimum of five generations. Cre-responsive Rosa26-loxP-eGFP reporter mice were generated as described van Berlo by crossing the dual-reporter R26-NZG line (FVB.Cg-Gt(Rosa)26Sort1(CAG-lacZ,EGFP)Glh/J; Jackson Labs #012429) to FLP-expressing B6.Cg-Tg(Pgk1-FLPo)10Sykr/J (Jackson Labs # 011065). Resulting R26-NG offspring were backcrossed to C57Bl/6J for a minimum of five generations to establish the line and then crossed with Kit-Cre-IRESGFPnls or Kit-MerCreMer mice to generate experimental animals. To induce Cre-mediated recombination in Kit-MerCreMer x R-GFP mice, animals were fed tamoxifen citrate chow (Teklad TD.130860) at a dose of 40mg/kg bw/d (400mg/kg diet) ad libitum for two weeks. Animals were switched back to normal lab chow for twenty-four hours prior to further procedures.

Animal Numbers
A total of 187 mice were used for this study. This includes wild-type C57BL/6 mice (n=173), Kit+/-cre x R-GFP transgenic mice (n=6) and their non-transgenic littermates (n=3), and Kit+/-MCM x R-GFP (n=5). To determine the maximum tolerated dose of isoproterenol, wild-type C57BL/6 mice (n=21) were used for dose titration, with animal receiving a single subcutaneous dose of 5 (n=5), 200 (n=5), 300 (n=1), 400 (n=1), 500 (n=1), 600 (n=1), 700 (n=1), 800 (n=1) or 1000 (n=5) mg/kg isoproterenol and their survival until 4 weeks after injection was assessed.

Of the wild-type C57BL/6 mice that received vehicle injection (n=39), 14 underwent serial echocardiography and were sacrificed at 1 day (n=4), 1 week (n=5) or 4 weeks (n=5) post-isoproterenol injection and hearts were perfusion-fixed and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice. The remaining animals (n=26) were dosed with saline and did not undergo serial echocardiography. Animals (n=9) were sacrificed at 1 day post-ISO injection for troponin I (n=5) measurements and for Western blot analysis of activated caspase 3 (n=4). Animals (n=4) were sacrificed at 1 day post-ISO injection, and hearts were embedded in Optimal Cutting Temperature Compound and frozen in liquid nitrogen for myocyte necrosis analyses using Evan’s blue dye. Animals (n=3) were sacrificed at 1 week post-ISO injection, and hearts were frozen in liquid nitrogen for molecular biology studies. Animals (n=9) were sacrificed at 4 week post-ISO and hearts were either frozen in liquid nitrogen for molecular biology (n=4), enzymatically digested (n=3) for myocyte isolation, or perfusion-fixed and paraffin-embedded for histology (n=2).

Of the wild-type C57BL/6 mice that received an injection of 200 mg/kg isoproterenol (n=45), 15 underwent serial echocardiography and were sacrificed at 1 day (n=5), 1 week (n=5) or 4 weeks (n=5) post-isoproterenol injection and their hearts were perfusion-fixed and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice. The remaining animals (n=30) were dosed with saline. Animals (n=9) were sacrificed at 1 day post-ISO injection for troponin I (n=5) measurements and for Western blot analysis of activated caspase 3 (n=4). Animals (n=5) were sacrificed at 1 day post-ISO injection, and hearts were embedded in Optimal Cutting Temperature Compound and frozen in liquid nitrogen for myocyte necrosis analyses using Evan’s blue dye. Animals (n=3) were sacrificed at 1 week post-ISO injection, and hearts were frozen in liquid nitrogen for molecular biology studies. Animals (n=9) were sacrificed at 4 weeks post-ISO and hearts were either frozen in liquid nitrogen for molecular biology (n=4), enzymatically digested (n=3) for myocyte isolation, or perfusion-fixed and paraffin-embedded for histology (n=2). Animals (n=3) underwent continuous ECG monitoring.

Of the wild-type C57BL/6 mice that received injection of 300 mg/kg isoproterenol (n=43), 14 underwent serial echocardiography and were sacrificed at 1 day (n=5), 1 week (n=5) or 4 weeks (n=4) post-
isoproterenol injection and their hearts were perfusion-fixed and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice. The remaining animals (n=29) were dosed with saline. Animals (n=8) were sacrificed at 1 day post-ISO injection for troponin I measurements (n=4) and for Western blot analysis of activated caspase 3 (n=4). Animals (n=5) were sacrificed at 1 day post-ISO injection, and hearts were embedded in Optimal Cutting Temperature Compound and frozen in liquid nitrogen for myocyte necrosis analyses using Evan’s blue dye. Animals (n=4) were sacrificed at 1 week post-ISO injection, and hearts were frozen in liquid nitrogen for molecular biology studies. Animals (n=10) were sacrificed at 4 weeks post-ISO and hearts were either frozen in liquid nitrogen for molecular biology (n=4), enzymatically digested (n=4) for myocyte isolation, or perfusion-fixed and paraffin-embedded for histology (n=2). Animals (n=2) underwent continuous ECG monitoring.

The wild-type C57BL/6 mice (n=5) that received vehicle injections twice daily for 6 consecutive days underwent echocardiography at baseline, 7 and 28 days with tissue Doppler imaging (TDI) and strain analysis, and were sacrificed at 28 days post-injections. The hearts were perfusion-fixed and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice. The wild-type C57BL/6 mice (n=9) that received 200mg/kg ISO injections twice daily for 6 consecutive days underwent echocardiography at baseline, 7 and 28 days with tissue Doppler imaging (TDI) and strain analysis, and were sacrificed at 28 days post-injections. The hearts were perfusion-fixed and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice.

During breeding of the c-kit-GFP transgenic animals, a total of 6 kit<sup>+/cre</sup>x R-GFP transgenic animals and 3 non-transgenic littermates. 7 of these animals received 200 mg/kg isoproterenol (4 kit<sup>+/cre</sup>x R-GFP transgenic and 3 non-transgenic) and underwent serial echocardiography. 2 transgenic mice served as a control and did not receive isoproterenol. All seven animals were sacrificed 4 weeks after injection. Of the transgenic kit<sup>+/cre</sup>x R-GFP transgenic mice were sacrificed at baseline and their hearts were perfusion digested for myocyte isolation to determine the percentage of GFP<sup>+</sup> myocytes present at baseline as previously described<sup>1</sup>. The hearts from the 3 non-transgenic animals and 2 transgenic animals were perfusion-fixed and paraffin-embedded for histology. The hearts of the remaining 2 transgenic females underwent perfusion digestion for myocyte isolation to determine the percentage of GFP<sup>+</sup> myocytes in each heart after isoproterenol injury. The body weight, heart weight and tibia length of all animals were recorded at sacrifice.

Kit<sup>+/MCM</sup>x R-GFP (n=5) were injected either with 200mg/kg ISO (n=3) or saline (n=2) twice daily for 6 consecutive days and were sacrificed at 28 days (n=5) post-isoproterenol injections and their hearts were cut in half in longitudinal sections. Half were frozen in liquid nitrogen and the other half fixed in formalin and paraffin-embedded for histology. The body weight, heart weight and tibia length of all animals were recorded at sacrifice.

Isoproterenol Dosing
Single-dose isoproterenol (ISO) was administered by subcutaneous injection into the loose skin (Ellison et al., 2013) over the neck. The maximum dose used in their publication was 200 mg/kg for mouse studies. For our study, we used 200mg/kg ISO and a higher dose of 300 mg/kg (as this was the highest tolerable non-lethal dose). Doses greater than or equal to 400 mg/kg were lethal within 30 min of injection. For the chronic ISO experiments, C57BL/6 mice were either injected s.c. with 200mg/kg ISO (n=10) or vehicle (n=5) twice daily for 6 consecutive days. For all experiments, a stock solution of 50 mg/mL isoproterenol (Sigma-Aldrich #I6504; St. Louis, MO) was prepared in sterile 0.9% saline. Dilutions made from thawed stock solution were prepared within 30 minutes of injection in sterile 0.9% saline to achieve concentrations of 200 and 300 mg/kg. All diluted isoproterenol solutions were kept on ice and away from light until injection.

Animal Procedures
EdU Minipump Implantation, Subcutaneous Isoproterenol Injections, Echocardiography and Cardiectomy. Animals were weighed, assigned treatment groups (vehicle, 200 or 300 mg/kg isoproterenol) and sacrifice date (1 day, 1 week or 4 weeks post-isoproterenol injection) prior to the start of experimental procedures to avoid selection bias, and isoproterenol doses were calculated based on the recorded body weights at baseline. Osmotic minipumps (Alzet; Cupertino, CA) were loaded with solution containing 39.0625 mg/mL EdU (Life Technologies, Carlsbad, CA) dissolved in 50/50% mixture of DMSO/ddH<sub>2</sub>O. Pumps were prepared as
previously described (Duran et al., 2014), then animals were anesthetized using isoflurane and the pumps were implanted subcutaneously between the two scapulae. The minipumps deliver a continuous infusion of EdU over the course of 1 week, and after 1 week all pumps were removed. Immediately following pump implantation (while the animal was still anesthetized), isoproterenol was injected into a subcutaneous depot just caudal to the site of pump implantation.

Permanent occlusion myocardial infarction was induced in 12-week old male C57BL/6 mice (n=4) (The Jackson Laboratory; Bar Harbor, ME) by ligation of the left anterior descending coronary artery (LAD) as described previously 2. Anesthesia was induced using 3% isoflurane and maintained at 1% (Abbott Laboratories; Chicago, IL). Mice were intubated after induction of anesthesia and ventilated at a rate of 180-190 breaths per minute and tidal volume of 250 μL. After 24 hours, animals were anesthetized and 1-1.5 mL of arterial blood was collected for troponin I analysis.

All animals underwent serial non-invasive transthoracic echocardiography with tissue Doppler imaging (TDI) and strain analysis under isoflurane anesthesia at 1 day (1d), 3 days (3d), 1 week (1wk), 2 weeks (2wk) and 4 weeks (4wk) after isoproterenol injection as previously described 3-6. Speckle-tracking based strain analyses were performed on long axis B-mode loops. Extensional strain of soft tissue in a predetermined direction can be defined as the change in length of a segment divided by its original length ([L1-L0]/L0), where strain rate (SR) is the rate of change of this deformation over time ([(L1-L0)/L0] x sec^-1) 7. Diastolic function was determined using ECHO coupled with pulsed Doppler and tissue Doppler imaging (TDI) techniques. From an apical long-axis view, transmitral flow velocities were recorded by setting the sample volume in the mitral orifice close to the tip of the mitral leaflets. Spectral waveforms were analyzed for peak early- and late-diastolic transmural velocities (E and A waves), E/A ratio and the acceleration time of the transmitral early diastolic peak flow velocity (EAT). EAT correlates well with the invasively measured LV relaxation parameters -dp/dt min, tau (g) and LVEDP 8. E´ was the tissue peak velocity obtained from LV posterior wall. Myocardial Performance Index (MPI), a non-invasive Doppler index for the assessment of overall cardiac function, is calculated as the sum of isovolumetric contraction and relaxation time (IVCT and IVRT) divided by the ejection time (ET) 9. At the assigned date of sacrifice, animals were anesthetized with inhaled isoflurane and cardiectomy was performed and the heart was rinsed and weighed. The aorta was then cannulated and the coronary arteries were cleared by perfusion with 1 mL cold Krebs-Henseleit Buffer. The heart was then arrested in diastole by perfusion with 1 mL of 100 mM cadmium chloride/1 M potassium chloride solution. The hearts were then gravity perfused with 30 mL 10% formalin at mean arterial pressure (100 mmHg). Fixed hearts were immersed overnight in 10% formalin and then stored in 70% ethanol for up to 1 week before being processed and embedded in paraffin wax blocks.

Continuous Electrocardiogram Monitoring
To determine the mechanism of death from high-dose isoproterenol (>400 mg/kg), animals were anesthetized using inhaled isoflurane and monitored with continuous electrocardiogram using leads placed subcutaneously on the ventral surface of both hind limbs and the right forelimb. After anesthetization, continuous ECG (3-lead ECG, ADInstruments) recording was begun using LabChart 7.3.7 (ADInstruments, Colorado Springs, CO), and animals were injected subcutaneously with 200 (n=3), 300 (n=2), 400 (n=3), or 1000 (n=3) mg/kg isoproterenol. In the high-dose groups (400 and 1000 mg/kg isoproterenol), ECG recording was continued until death (after 5min of asystole). Animals dosed with 400 and 1000 mg/kg expired within 25 minutes. Animals dosed with the nonlethal dose of 200 or 300 mg/kg underwent ECG monitoring for 1 hour (long enough to detect lethal arrhythmias at the higher/lethal doses). The animals treated with 200 or 300 mg/kg were sacrificed immediately after ECG recording.

Arterial Cardiac Troponin Analysis
To detect the presence of serum markers of cardiac injury, animals were dosed with vehicle (n=5), or 200 (n=5), or 300 (n=4) mg/kg isoproterenol. Myocardial infarction was induced in C57BL/6 mice (n=4), which served as a positive control. After 24 hours, animals were anesthetized and 1-1.5 mL of arterial blood was collected by inferior vena cava puncture. Blood was placed in lithium carbonate-coated vacutainers, samples were microcentrifuged at 1300 rpm for 10min, and serum samples were sent to Antech Diagnostics (Lake Success, NY) for cardiac troponin I analysis.

Single cell isolation
Mouse cardiac myocytes were isolated as previously described 5,6,10,11. Briefly, the aorta was cannulated and perfused retrograde to enzymatically digest the heart. When softened, the atria were removed and the
ventricles were mechanically dissociated to obtain single cells. The cell solution was exchanged with a BSA containing solution and allowed to settle. Next, the cells were washed in normal tyrode’s solution and then fixed with 4% paraformaldehyde in PBS. Care was taken between solution exchanges to minimize myocyte loss. Following enzymatic digestion, LV myocytes were fixed in paraformaldehyde (4% in PBS, Affymetrix) and nuclei stained with Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies; Eugene, OR) and DAPI (Calbiochem). Total number of myocytes was determined by hemocytometer and Edu+ myocytes were counted using a fluorescent microscope (Nikon eclipse Ti). Images were collected. Surface area and nuclei distribution were evaluated using ImageJ software. On average ~ 150 myocytes per mouse were analyzed from 10 mice.

Calcium transients and contraction measurements: After isolation, myocytes from transgenic Kit+/Cre x R-GFP mice (n=2) were loaded with 5-10 uM indo-1 in normal tyrode’s, as described previously12. Myocytes were paced at 1HZ and sarcomere length was recorded with an Ion Optix system. Calcium transients were obtained by indo-1 fluorescence intensity ratio at 410nm to 480 nm. 3-4 GFP+ and GFP-myocytes from 2 mice were used. Myocyte fractional shortening was not different between indo-1 loaded and unloaded cells. A total of 300,000 cells were counted for quantification of eGFP fluorescent myocytes.

Myocyte Necrosis Analysis
To quantify the degree of myocyte necrosis, male C57BL/6 mice (n=14) received intraperitoneal injection of 50µl/10g body weight Evans Blue (Sigma-Aldrich; St. Louis, MO). To determine Evan’s blue dye (EBD) uptake in cardiomyocytes, mice were injected with EBD (20mg/ml PBS; 50µl/10g body weight). After 24 hours, animals received injection of vehicle (n=4), 200 mg/kg (n=5) or 300 mg/kg (n=5) isoproterenol, and then after 24 hours, animals were sacrificed. Hearts were harvested, embedded in Optimal Cutting Temperature Compound (O.C.T, Tissue-Tek; Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Tissues were cut into 7 micron sections and counterstained with wheat germ agglutinin conjugated with fluorescent isothiocyanate (FITC; Sigma-Aldrich; St. Louis, MO) for one hour at room temperature to visualize membranes (green) versus EBD (which fluoresces red). Images were taken on a Nikon Eclipse Ti-S inverted microscope equipped with NIS elements Advanced Research (AR) imaging software (Nikon Instruments Inc.; Melville, NY) to determine the percentage of EBD-positive cardiomyocytes.

Bright Field Histology and Immunohistochemistry
Paraffin-embedded samples fixed at 4 weeks post-injection were stained with Masson’s Trichrome (Sigma-Aldrich; St. Louis, MO) for myocyte cross-sectional area and bioquantification of fibrosis. Masson’s Trichrome-stained bright field micrographs were acquired using a light microscope (Olympus BX40, Spot Software 5.1.29; Sterling Heights, MI). Myocyte cross-sectional area was measured using NIH Image J software as previously described13. A total of 4410 myocytes were counted from 9 animals. Bioquantification of the percent fibrotic area of myocardium was determined using a Nikon microscope (Mellville, NY) interfaced with an analog camera and a bioquantification software system (Bioquant TCW 98; Nashville, TN). A total of 54 short-axis cross-sections from 9 mice and 24 longitudinal sections from 6 mice were analyzed.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed on paraffin-embedded tissues fixed at 24 hours after isoproterenol injection. Samples were then immunostained to quantify the number of apoptotic myocytes. The DeadEnd Fluorometric TUNEL system was used to label and immunostain apoptotic nuclei (Promega #G3250; Madison, WI). Immunostaining for cardiac contractile proteins was performed using mouse IgG anti-sarcomeric tropomyosin (Sigma-Aldrich #T9283; St Louis, MO), and the secondary antibody Rhodamine Red-X donkey anti-mouse IgG (715-295-150) was used to detect IgG anti-sarcomeric tropomyosin. The identification of cardiomyocytes and quantification of Tunel positive/negative cells was done semi-automatically using Nuquantus, a Matlab toolbox for machine learning based detection and quantification of cells of interest in complex fluorescent tissue images14. The patterns needed to detect cardiomyocytes were trained in Nuquantus using a reference dataset of mouse and swine cardiomyocytes labeled by an expert histopathologist. These patterns were then used to automatically detect valid cardiomyocytes in the Tunel study dataset. The automatic detection was then checked for quality control by the histopathologist analyst to remove false positive detection errors. A total of 6846 myocyte nuclei were counted for TUNEL analysis.

For immunostaining and EdU detection, the Click-it EdU Alexa Fluor 488 Imaging Kit (Life Technologies; Eugene, OR) was used along with primary mouse IgM anti-α-sarcomeric actin (Sigma-Aldrich #A2172; St. Louis, MO) with secondary rhodamine red-X donkey anti-Mouse IgM (Jackson
Immunoresearch Laboratories #715-295-020; West Grove, PA) for detection of mouse IgM anti-α-sarcomeric actin. More than 34,000 myocyte nuclei and more than 45,000 non-myocyte nuclei from 9 animals were counted for analysis.

For detection of GFP in paraffin-embedded hearts from transgenic Kit^+Cre x R-GFP mice (n=2) and Kit^+MCM x R-GFP (n=5) mice, sections were stained with primary chicken anti-GFP antibody (Life Technologies # A10262; Eugene, OR) and secondary FITC donkey anti-Chicken IgG (Jackson Immunoresearch Laboratories #703-095-155; West Grove, PA). Immunostaining for cardiac contractile proteins was performed using with primary mouse IgM anti-α-sarcomeric actin (Sigma- Aldrich #A2172; St. Louis, MO) and secondary rhodamine red-X donkey anti-Mouse IgM (Jackson Immunoresearch Laboratories #715-295-020; West Grove, PA) for detection of mouse IgM anti-α-sarcomeric actin. A total of 32 cross-sections from 2 Kit^+Cre x R-GFP mice and 87 longitudinal sections from 5 Kit^+MCM x R-GFP were analyzed for GFP+ cardiomyocytes. Paraffin-embedded hearts from vehicle injected C57BL/6 mice were stained against GFP, which served as control.

For all immunostains, nuclei were labeled with 4',6- diamidino-2-phenylindole (DAPI, Millipore; Billerica, MA). Confocal micrographs of all immunostains were acquired using a Nikon Eclipse T1 confocal microscope (Nikon Inc.; Mellville, NY).

Real-time polymerase chain reaction (PCR)
Hearts were harvested from vehicle (n=3), 200mg/kg (n=4), and 300mg/kg (n=4) group at 1 week post injection and at 4 week post injection (veh (n=4), 200mg/kg (n=4), 300mg/kg (n=4)). RNA was extracted from mouse hearts with TRizol Reagent. The RNA was cleaned using the Quick-RNA™ MiniPrep (Zymo Research) clean-up protocol. Reverse transcription (RT) reaction was performed using the SuperScript III first strand synthesis system for RT-PCR (Invitrogen) and oligo-dt primers according to the manufacturer’s instructions. Real-time PCR was performed using the Quantifast Sybrgreen PCR kit (Qiagen). Data generated from mouse heart samples were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following primer sets were used (forward, reverse): GAPDH 5'-ACTGAGCAAGAGAGGCCTTA, 5'-TATGGGGGCTCCTGGGATGGAA; atrial natriuretic factor (ANF) 5'-GCCTCTGAGTGAAGCAGACTG, 5'-GGAAGCTTGGTTGAGAGCCTA; brain natriuretic factor (BNP) 5'-CTCTGGGACCTGTAAGAGGA, 5'-AGTCAAGAAACTGGAAGTCTCC; alpha myosin heavy chain (αMHC) 5'-ACCTACACAGACAGAGGAA, 5'-ATTGTGTATTGGCCACACCG; beta myosin heavy chain (βMHC) 5'-ACCTACACAGACAGGAA, 5'- TTGCAAAGAGTCCAGGTCTGAG.

Western Analysis
Lysates from heart tissue (n=12) or HEK cells were prepared and analyzed using Western analysis as previously published. The following primary antibodies were used: total caspase 3 (Cell Signaling #9662, Beverly, MA, 1:1000) that detects total and cleaved Caspase 3, activated Caspase 3 (Cell Signaling #9661; Beverly, MA, 1:1000), and glyceraldehyde-3- phosphate dehydrogenase (GAPDH; AbD Serotec #MCA4739G; Kidlington, UK, 1:1000). The following secondary antibodies were used: 800CW Donkey anti-Rabbit (#926-32213) and 680RD Donkey anti-Mouse (#926-68072) purchased from LICOR (Lincoln, NE, 1:10,000).

Statistical Analysis
All data analyses were performed using SAS version 9.3. Data are shown as mean ± SEM. For variables such as ECHO parameters (e.g., ECHO-derived cardiac function and volumes, global and apical longitudinal strain data) that were measured repeatedly over time at baseline (BL) and 1, 3, 7, 14, and 28 days post isoproterenol (ISO) injection, a mixed-effects model approach was employed to study the differences in each variable between three groups (vehicle, 200mg/kg ISO, and 300mg/kg ISO) at each time point as well as the overtime changes at the 5 time points compared to baseline within each of the three groups. This modeling approach takes into account the within-animal correlation among repeated measurements overtime via a flexible structure such as ARH(1) or AR(1) for variance components, affording different magnitude of variability across the three groups. This is especially important in our study as it is often evident that there exists heterogeneity between the three experimental groups. Furthermore, this modeling technique allows us to conveniently test under the unified mixed-effects model framework the 3 pairwise group differences at each timepoint or 5 overtime changes compared to baseline within each of the three groups simultaneously via the Bonferroni or Dunnett-Hsu adjustment, respectively. In addition, to guard against a potentially overinflated type I error rate, p<0.05/5=0.01 was used for between-group comparisons at the 5 post injection timepoints.
For variables that were measured only once post ISO injection (e.g., the percentages of EBD positive myocytes, TUNEL+ myocyte and non-myocyte nuclei, and Tn-I levels measured at day 1 post-injection), the overall group comparison was tested using the Kruskal-Wallis test or ANOVA F-test, when appropriate, while the pairwise group comparisons were performed using the exact Wilcoxon rank-sum test or unequal variance two-sample t-test. Due to the relatively small group sizes, however, this part of the data analyses was not adjusted for multiplicity.
Online Figure 1: ECG traces after ISO injection. Related to isoproterenol dosing and heart rate during ECHO

A. Representatives ECG traces after s.c. injection of lethal doses of isoproterenol (1000mg/kg and 400mg/kg isoproterenol), 7 min after injection, ECG showed sinus arrest with a ventricular escape rhythm (1000mg/kg), or with premature ventricular contractions (400mg/kg) which ended with lethal tachyarrhythmias. B, Heart rate during echocardiography. Heart rate did not significantly differ between groups at any time point. NS=not significant (p>0.05). B, Data are shown as mean ± SEM.
Online Figure II: Diastolic function post-ISO injection. Related to Figure 1

ECHO measurements were performed at baseline (BL), 1, 3, 7, 14, 28 days post-injection (vehicle (n=14), 200mg/kg ISO (n=15) and 300mg/kg ISO (n=14)). A-D. Diastolic function was determined using ECHO coupled with pulsed wave Doppler (PW) and tissue Doppler (TDI) techniques. A-D. Spectral waveforms were analyzed for peak early- and late-diastolic transmitral velocities (E and A waves), E/A ratio (B) and the acceleration time of the transmitral early diastolic peak flow velocity (E_AT) (D). C. E’ was the tissue peak velocity obtained from LV posterior wall. There was no difference in diastolic function (E, E/A, E’, E_AT) between groups at any time point. *p<0.05 vs BL vehicle. NS=not significant (p>0.05). Data are shown as mean ± SEM. E. Representative spectral waveforms at baseline (upper panel) and 1 day post-vehicle, 200mg/kg ISO, or 300mg/kg ISO injection (lower panel). S=systole.
Online Figure III: Western analysis
Measuring total Caspase-3 or activated Caspase-3 after cleavage in heart lysates after treatment with vehicle, 200mg/kg ISO, or 300mg/kg ISO. Two primary antibodies were used: (1) total caspase-3, which detects total and cleaved Caspase-3, and (2) activated Caspase-3. Positive control (+) is lysate from HEK cells treated with 2mmol/L H$_2$O$_2$ for 24 hours.
Online Figure IV: EdU+ nuclei after myocardial infarction. Related to Figure 5
Representative images of EdU+ nuclei in the infarct area (A), border zone (B), and remote zone (C) 4 weeks post-MI induced by permanent occlusion of the LAD. Tissue sections were immunostained against α-sarcomeric actin (red), 5-ethynyl-2'-deoxyuridine (EdU, green), and nuclei labeled with 4',6-diamidino-2-phenylindole (DAPI, blue). A significant number of EdU+ non-myocyte nuclei were observed in the injured border zone and infarct area with fewer in the viable tissue of the remote zone. These likely are inflammatory cells that infiltrate into the injured heart after MI. Scale bars=50µm.
Online Figure V: Cardiac function in transgenic kit+cre x R-GFP mouse post-ISO injection. Related to Figure 7

ECHO measurements were performed at baseline (BL), 1, 3, 7, 14, 28 days post-injection. C57BL/6 mice (n=14) were injected with vehicle, kit+cre x R-GFP mice (ckit GFP+, n=4) and the non-transgenic littermates (ckit GFP−, n=3) with 200mg/kg isoproterenol (ISO). #p<0.05 between groups. Data are shown as mean ± SEM.
Online Figure VI: Cardiac function, heart weight to body weight ratio and myocyte cross-sectional area after chronic ISO administration. Related to Figure 8

A-D, ECHO measurements in C57BL/6 mice were performed at BL (baseline), 1 and 4 weeks post-injections (veh (n=5), ISOchron (n=9)). A and B, Longitudinal strain and strain rate showed reduced contractility in the ISO group at 1 and 4 weeks post-injection. C, The myocardial performance index (MPI), a non-invasive Doppler index for the assessment of overall cardiac function, was significantly increased at 1 week post-ISO injections, indicating an impairment in global cardiac function. D, E/E’, a surrogate for left ventricular filling pressures, was slightly increased at 1 and 4 weeks post-ISO injections. E, Representative spectral waveforms of peak early- (E) and late-diastolic (A) transmitral velocities in a vehicle and an ISO-treated C57BL/6 mouse. F, Dose-dependent fold change increase in...
the percentage of EdU+ non-myocyte nuclei after veh (n=8), single 200mg/kg ISO (n=3), single 300mg/kg ISO (n=3) and multiple (twice daily for 6 days) ISO (n=5) injections in C57BL/6 mice. G-J, In C57BL/6 mice, myocyte cross-sectional area (CM CSA) (G) and heart weight to body weight ratio (HW/BW) (H); in Kit$^{+/MCM}$ x R-GFP mice, CM CSA (I) and HW/BW (J). K, Bright-field micrographs of a Masson’s trichrome-stained tissue section from a Kit$^{+/MCM}$ x R-GFP mouse at 4 weeks post-ISO injection. Scale bar=200µm. L, Histological analysis of eGFP+ cells from spleen, lung and intestine from Kit$^{+/MCM}$ x R-GFP mice that were given tamoxifen for two weeks prior to chronic ISO dosing. Nuclei are shown in blue and green shows eGFP+ cells in the expected patterns coincident with known regions of c-kit protein expression. Scale Bars=100µm. *p<0.05, **p<0.01 and ***p<0.001 vs. BL ISO, #p<0.05 between groups, @p<0.05 and @@@p<0.001 vs. veh, NS=not significant (p>0.05). A-D and F-J, Data are shown as mean ± SEM.
Supplemental References


Legends for Video files

**Online Video I:**
Transthoracic ECHO loops at 1 day post-injection of 300mg/kg ISO. Related to Figure 1
Parasternal short axis view showing increased cardiac function.

**Online Video II:**
Transthoracic ECHO loops at 1 day post-injection of 300mg/kg ISO. Related to Figure 1
Parasternal long axis view showing increased cardiac function.

**Online Video III:**
Transthoracic ECHO loops at 1 day in a vehicle treated mouse. Related to Figure 1
Parasternal short axis view showing normal cardiac function.

**Online Video IV:**
Transthoracic ECHO loops at 1 day in a vehicle treated mouse. Related to Figure 1
Parasternal long axis view showing normal cardiac function.