Impaired oxidative metabolism and calcium mishandling underlie cardiac dysfunction in a rat model of post-acute isoproterenol-induced cardiomyopathy

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Willis BC, Salazar-Cantú A, Silva-Platas C, Fernández-Sada E, Villegas CA, Ríos-Argaiz E, González-Serrano P, Sánchez LA, Guerrero-Beltrán CE, García N, Torre-Amione G, García-Rivas GJ, Altamirano J. Impaired oxidative metabolism and calcium mishandling underlie cardiac dysfunction in a rat model of post-acute isoproterenol-induced cardiomyopathy. Am J Physiol Heart Circ Physiol 308: H467–H477, 2015. First published December 19, 2014; doi:10.1152/ajpheart.00734.2013.—Stress-induced cardiomyopathy, triggered by acute catecholamine discharge, is a syndrome characterized by transient, apical ballooning linked to acute heart failure and ventricular arrhythmias. Rats receiving an acute isoproterenol (ISO) overdose (OV) suffer cardiac apex ischemia-reperfusion damage and arrhythmia, and then undergo cardiac remodeling and dysfunction. Nevertheless, the subcellular mechanisms underlying cardiac dysfunction after acute heart damage subsides are not thoroughly understood. To address this question, Wistar rats received a single ISO injection (67 mg/kg). We found in vivo moderate systolic and diastolic dysfunction at 2 wk post-ISO-OV; however, systolic dysfunction recovered after 4 wk, while diastolic dysfunction worsened. At 2 wk post-ISO-OV, cardiac function was assessed ex vivo, while mitochondrial oxidative metabolism and stress were assessed in vitro, and Ca2+ handling in ventricular myocytes. These were complemented with sarcoplasmic reticulum Ca2+-ATPase (SERCA), phospholamban (PLB), and RyR2 expression studies. Ex vivo, basal mechanical performance index (MPI) and oxygen consumption rate (MVO2) were unchanged. Nevertheless, upon increase of metabolic demand, by β-adrenergic stimulation (1–100 nM ISO), the MPI versus MVO2 relation decreased and shifted to the right, suggesting MPI and mitochondrial oxidative damage, and secretion of pro-inflammatory cytokines such as TNF-α, necrosis, and apoptosis (19, 33, 65). Additionally, because catecholamines are highly unstable, they are a potent source of reactive oxygen species (ROS), which may exacerbate the damage by ischemia-reperfusion (34).

Some studies suggest that once the ischemic phase has passed, ISO-OV hearts undergo major chronic remodeling (57, 65) and, after 2 wk, become hypertrophic and dilated and develop dysfunction (13, 37, 57), and these alterations are proportional to the original ISO dose (52, 57). Electrocardiographic disturbances, fibrosis, and enhanced TNF-α may even persist after 9 wk of ISO-OV, suggesting a slow recovery process (65); however, recently it was suggested that the acute ISO effects are reversible below a certain threshold dose (between 50 and 100 mg/kg) (52). Nevertheless, the subcellular mechanisms involved in cardiac dysfunction, after both acute ischemic and oxidative damage have subsided, are still unclear (13). Although mitochondrial and intracellular Ca2+ handling dysfunction have been implicated (18), those have not been thoroughly studied. Therefore, our main objective was to determine whether there is a pathophysiological link between mitochondrial dysfunction, contractility, and Ca2+ mishandling that could underlie cardiac performance impairment in the context of SIC. For this we sought out to characterize cardiac function in vivo at 2 and 4 wk after ISO-OV. We also assessed ex vivo cardiac performance, mitochondrial function, and cellular Ca2+ handling at 2 wk post-ISO-OV, under basal conditions and upon increased metabolic cardiac demand (in response to physiological β-adrenergic stimulation). We tested the hypothesis that persistent mitochondrial dysfunction would impair ATP production, not only required for fueling myofila-
ments contraction (28) but active Ca\(^{2+}\) transport [i.e., by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)] as well (39). Furthermore, given the recent evidence of functional modulation of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel (RyR2) by mitochondrial-generated ROS (10, 15, 66), SR Ca\(^{2+}\) release could also be compromised. Altering SR Ca\(^{2+}\) release and reuptake would affect strength and relaxation of cardiac contraction (26, 41) and underlie Ca\(^{2+}\)-linked arrhythmias (1, 44, 45).

We found that 2 wk after a single ISO injection (67 mg/kg) in rats, the heart was in a state of systolic and diastolic contractile dysfunction; nevertheless, systolic performance partially recovered at 4 wk, whereas worsening diastolic dysfunction persisted. Importantly, at 2 wk, physiological β-adrenergic response was impaired at the cellular and whole heart level. Furthermore, at the same time we found mitochondrial dysfunction and organelle fragility, accompanied by enhanced oxidative stress. Concomitantly, diastolic and systolic Ca\(^{2+}\) signaling were profoundly affected; with increased spontaneous Ca\(^{2+}\) spark frequency and smaller Ca\(^{2+}\) transients, with slower decay, and lower SR Ca\(^{2+}\) content. Importantly, Ca\(^{2+}\) signaling alterations were not due to changes in SERCA, phospholamban (PLB), or RyR2 expression and probably the enhanced RyR2 resting activity was not due to PKA-dependent phosphorylation either, since both basal cAMP levels and PKA

**METHODS**

**Solutions and reagents.** All chemical reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO), unless otherwise stated.

**ISO-OV murine model.** All studies were approved by the Institutional Animal Care and Use Committee of the School of Medicine of the Tecnológico de Monterrey (Protocol 2011-009). Ninety male Wistar rats of 250 - 350 g were injected with a single subcutaneous dose of ISO (ISO hydrochloride; 67 mg/kg body wt) in a sterile saline solution to induce myocardial injury (13, 18). As previously reported with this ISO dose (13, 57), we observed ~20% acute mortality within the first 48 h, and following this acute period, all ISO-treated rats survived the 4 wk, during which all studies were performed. Age-paired rats injected with saline solution served as controls. Both groups were fed standard rat chow and water ad libitum.

**Noninvasive determination of cardiac function.** Noninvasive, transcatheter cardiac ultrasonography was performed according to the procedures previously described (11, 27) to evaluate cardiac function at baseline (before ISO-OV) and after 2 and 4 wk in ISO-OV animals. Briefly, animals were anesthetized with pentobarbital sodium (40 mg/kg body wt ip) and placed on top of a custom-made electric blanket for temperature control (37°C). A Philips EnVisor (Philips Healthcare, Andover, MA) ultrasound recorder equipped with a 12 MHz S-type transducer was used to determine systolic and diastolic function. Cine loop recordings (3 s) were acquired in parasternal long axis, parasternal short axis, and four chamber windows, respectively, and were analyzed by a second blinded individual. Animals were placed in supine position for parasternal long axis cineloop recording [where left ventricle (LV), length, and aortic outflow tract diameter were measured] and parasternal short axis cineloop recording [where, in M-mode line scan at the level of the papillary muscles, LV, interventricular septum, and LV posterior wall, and systolic and diastolic measurements were obtained]. Afterward animals were placed in the left lateral decubitus position for apical four-chamber cineloop recording (where transmitial and transaortic wave velocities were obtained by placing a pulsed Doppler wave sample volume at the tip of the mitral valve inflow and aortic outflow tracts, respectively). LV systolic function was measured as ejection fraction (EF) [LV telediastolic volume (LV TDI) – LV telesystolic volume (LV TSV)/LV TDI × 100] and fractional shortening (FS) [diastolic LV internal diameter (LVID) – systolic LVID/diastolic LVID × 100]. To determine LV diastolic function, transmitial early (E) and late/atrial (A) wave velocities (E/A) ratio and “W” wave interval corrected isovolumetric relaxation time (IVRT/R-R) were recorded. LV TSV and LV TDI were estimated using a modified Simpson method. Finally, other hemodynamic parameters such as HR (60/R-R interval), stroke volume (SV) [Aortic (Ao) time velocity integral × Ao cross sectional area (CSA)], and cardiac output [HR × SV] were calculated.

**Ex vivo mechanical performance and oxygen consumption.** Rats were anesthetized with pentobarbital sodium (80 mg/kg body wt ip). Following thoracotomy, hearts were quickly excised, mounted on a Langendorff apparatus, and perfused retrogradely at a constant flow rate of 12 ml/min with a Krebs-Henseleit (K-H) buffer. K-H buffer composition was (in mM) 125 NaCl, 5.4 KCl, 1.0 MgCl\(_2\), 0.5 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 2.5 CaCl\(_2\), 11 glucose, and 0.1 octanoate. This solution was kept at 37°C, continuously agitated and bubbled with 95% O\(_2\)-5% CO\(_2\) gas (17). A latex balloon connected to a pressure transducer was inserted into the left ventricle and filled with saline solution. The pulmonary artery was cannulated and connected to a closed chamber (Gilion Medical Electronics, Middleton, WI) to measure O\(_2\) concentration in the coronary effluent with a Clark-type oxygen electrode (YSI Life Sciences, Yellow Springs, OH). The rate of myocardial O\(_2\) consumption (MVO\(_2\)) was calculated as the difference between O\(_2\) concentration in the K-H buffer before (100%) and after perfusion. All variables were recorded using a computer acquisition data system (Data-Trax, World Precision Instruments, Sarasota, FL). Baseline was established in K-H buffer and thereafter hearts were perfused with ISO at 1, 10, and 100 nM MVO\(_2\) and the myocardium performance index (MPI) were obtained from the last 3 min of contraction at each ISO concentration. MPI was defined as the product of LV developed pressure × HR (LVDP × HR, mmHg × heart beats × min\(^{-1}\)).

**Ventricular myocyte isolation.** Cells were dissociated according to a method previously described (22). Briefly, hearts were removed and mounted on a Langendorff apparatus and then perfused with collage-nase-containing solution (1 mg/ml collagenase type II; Worthington Biochemical, Lakewood, NJ) at 37°C. Thereafter, ventricles were dissected and cells mechanically disaggregated. Cells were kept in normal Tyrode (NT) solution at room temperature and used within 6 h. NT solution contained (in mM) 140 NaCl, 4 KCl, 1 MgCl\(_2\), 10 HEPES, 10 glucose, and 1 CaCl\(_2\) (pH 7.4) adjusted with NaOH.

**Mitochondrial isolation.** Mitochondria were isolated as previously reported (55). Briefly, excised hearts were digested with 0.3 mg/ml of protease Nagarse for 10 min and homogenized in a buffer solution containing (in mM) 10 HEPES 10, 25 sucrose, and 1 EDTA, and pH was adjusted at 7.2. Then, by differential centrifugation, mitochondria were isolated and suspended in EDTA buffer solution. Mitochondrial
protein was determined by the Lowry method, and yield was determined by dividing protein density by the total grams of heart tissue.

**Mitochondrial respiration.** Isolated mitochondria were suspended (0.5 mg/ml) in respiration buffer (RB) containing (in mM) 125 KCl, 5 KH₂PO₄, 10 HEPES, and pH adjusted at 7.2 with KOH. Mitochondrial O₂ consumption was determined with a Clark-type electrode. State 4 respiration rate was evaluated as Succinate-linked respiration, in the presence of 10 mM succinate (substrate for respiratory complex II) plus 1 µg/ml rotenone (a respiratory complex I inhibitor), or as NADH-linked respiration, measured in the presence of 5 mM glutamate-malate (substrate for respiratory complex I). State 3 respiration rate was measured after addition of 200 µM ADP. The respiratory control ratio was expressed as the state 3/state 4.

**Mitochondrial Ca²⁺ retention capacity.** Ca²⁺ retention capacity is a functional assessment of the sensitivity of the mitochondrial permeability transition pore (mPTP) opening to mitochondrial Ca²⁺ overload (3) and was evaluated by monitoring the absorbance at 675–685 nm (Synergy Biotek, Winooski, VT) of the metallochromic Ca²⁺ indicator Arsenazo III outside the mitochondria, as previously described (47). Briefly, isolated mitochondria (0.5 mg/ml) were suspended and incubated for 3 min in RB with added (in mM) 10 succinate, 0.2 ADP, 0.05 CaCl₂, 0.03 Arsenazo III, and 1 µg/ml rotenone. Thereafter, steps of 10 nmol CaCl₂ were added every 3 min. Ca²⁺ was rapidly taken up by the mitochondria, resulting in extra-mitochondrial Ca²⁺ decrease near to baseline. After a Ca²⁺ loading threshold, extra-mitochondrial Ca²⁺ concentration abruptly increased, indicating a massive release of mitochondrial Ca²⁺ due to mPTP opening. The amount of CaCl₂ necessary to trigger this Ca²⁺ release was used as indicator of mPTP susceptibility to Ca²⁺ overload (17). Cyclosporine A (0.5 µM), a potent calcineurin inhibitor, added before the first CaCl₂ pulse was used as control.

**Mitochondrial oxidative stress.** Aconitase enzyme activity (sensitive to superoxide anion O₂⁻) was measured by the addition of 150 µg of mitochondrial protein to 1 ml of medium base containing (in mM) 120 KCl, 5 KH₂PO₄, 10 HEPES, 1 EGTA, 5 MgCl₂, 0.01% Triton X-100, 0.2 NADP, 5 citrate, and 2 U. i. isocitrate dehydrogenase (pH 7.2). Excitation and emission wavelengths were 340 and 460 nm, respectively. NAPDH production was estimated by using a NADPH calibration curve and expressed in units of activity. Enzyme activity was normalized with the citrate synthase activity, assessed in a medium containing 100 mM KH₂PO₄, 1 mM oxaloacetate, acetyl-CoA, 30 µM DTNB [5–5’-Dithiobis-(2-nitrobenzoic acid)], and 0.05% Triton X-100 (pH 7.4), and 150 µg of mitochondrial protein, measuring of CoA with thiol (CoA-SH) formation in the presence of DTNB, which reacts with thiol forming TNB-SH-CoA (5-thio-2-nitrobenzoic acid) monitored at 412 nm. The units of activity were estimated with the extinction coefficient of TNB (13.2 mM cm⁻¹).

Ca²⁺ transients, sparks, and SR Ca²⁺ content in intact myocytes. Isolated cells were incubated for 30 min in NT solution containing Fluo-4 AM (5 µM) (Life Technologies, Carlsbad, CA) and afterward washed with a fluorophore-free solution. Fluo-4-loaded cells were plated on laminin-covered glass coverslips mounted in a superfusion chamber. All fluorescence measurements were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a D-apochromatic 63×, 1.2 NA, oil objective. An Argon laser was used to excite the fluorophore at 488 nm and emission collected at 500–600 nm. Linescan images were recorded along the longitudinal axis of the cell at 500 Hz, with a pixel size of 100 nm and pinhole optimized for a resolution of 0.4 µm in the focal plane and <1 µm in the z-axis. For Ca²⁺ transients, cells were field-stimulated at 1 Hz (MYP100 MyoPacer Field Stimulator; IonOptix, Milton, MA) to attain steady state SR Ca²⁺ content and the last paced event was recorded along with the subsequent 2 to 3 s for spontaneous Ca²⁺ sparks recording. This protocol was repeated at 3, 5, and 10 min after perfusion with NT solution containing ISO (100 nM), for both the control and ISO-OV cells. Fluorescence data were normalized as F/F₀, where F is fluorescence intensity and F₀ is average fluorescence at rest. Ca²⁺ sparks were detected and characterized following established criteria (14) using an algorithm developed for ImageJ (http://imagej.nih.gov/ij/; NIH, Bethesda, MD) (40). SR Ca²⁺ content was estimated from the ΔF/F₀ of the cytosolic Ca²⁺ signal evoked by rapid caffeine (10 mM) application following a train of 5 field stimulation pulses (5).

**cAMP and PKA activity assay.** Heart homogenates were obtained from ventricles in lysis buffer containing (in mM) 20 MOPS, 50 β-glycerolphosphate, 50 NaF, 1 sodium vanadate, 5 EGTA, 2 EDTA, 1 DTT, 1 benzamidine, 1 PMSF, 1% Nonidet P-40, 10 mg/ml leupeptin, and aprotinin, using a polytron at 10,000 rpm. After centrifugation at 11,000 g the supernatant was collected. cAMP levels were determined with the cAMP complete ELISA kit (ENZO Life Sciences, Farmingdale, NY) following manufacturer’s instruction. PKA activity was determined using a PKA kinase activity kit (ENZO Life Sciences) using 200 and 400 ng/well of the phospho-specific substrate antibody and the anti-rabbit IgG HRP conjugate, respectively.

**Western blot assay.** Heart ventricular tissue was isolated from all experimental groups and flash-frozen in liquid nitrogen. Tissue was homogenized, and proteins were extracted in RIPA buffer (Cell Signaling Technology, Danvers, MA). Tissue (30 µg) was resolved on 7–15% gel via SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with rabbit, goat, and mouse antibodies specific for PLB (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), SRCA (1:500; Santa Cruz), RyR2 (1:2,000; Thermo Fisher, Waltham, MA), and GAPDH (1:1,000; Abcam, Cambridge, MA) and subsequently probed with donkey anti-rabbit, anti-goat, and anti-mouse (1:10,000; Santa Cruz), respectively. Western blots were developed with Clarity TM Western ECL (Bio-Rad, Hercules, CA) and quantified by using a BioSpectrum 415 imaging system (UVP, Upland, CA).

**Statistical analysis.** Data are expressed as means ± SE. Unpaired Student’s t-test and one-way ANOVA with Bonferroni adjustment were performed when appropriate to compare experimental groups. A statistically significant difference was considered when P < 0.05. Data processing and statistical tests were carried out with Microsoft Excel (2007; Redmond, WA) and GraphPad Prism (V. 2.0; La Jolla, CA). Graphs were generated using Systat Sigma Plot (V. 11; San Jose, CA).

**RESULTS**

**In vivo cardiac systolic and diastolic function.** Table 1 shows pooled data for the non-invasive transthoracic echocardiographic parameters at 2 wk after ISO-OV injection. ISO-OV hearts had significant diastolic dysfunction, as evi
duced by 10.220.33.2 on October 15, 2017 http://ajpheart.physiology.org/ Downloaded from
Evidence by a ~20% decrease in the E/A ratio when compared with controls \( (P < 0.05) \). Several ISO-OV hearts developed profound apical ballooning with a hyper contractile base (Supplement Videos S1 and S2). Interestingly, there was mild, but significant, systolic dysfunction in ISO-OV hearts than in controls (notice the small decreases in FS and EF; \( P < 0.05 \)); however, both LV TSV and LV TDV were significantly increased after ISO-OV (93% and 33%, respectively). There was no statistical difference in IVRT/R-R, HR, SV, and CO in post-ISO-OV rats compared with controls at 2 wk. Figure 1 shows the normalized changes in systolic and diastolic function over time after ISO-OV. As shown in Fig. 1A, after 4 wk post-ISO-OV systolic function partially recovered, as evidenced by an increase in both IVRT/R-R (from 102 ± 4 to 105 ± 4%, at 2 and 4 wk, respectively; \( P < 0.05 \)) and LV TDV (from 134 ± 8% to 266 ± 55% of baseline, 2 and 4 wk, respectively; \( P < 0.05 \)) and a decrease in E/A ratio (from 84 ± 5% to 64 ± 7%, 2 and 4 wk, respectively; \( P < 0.05 \)).

Ex vivo cardiac contractility and oxygen consumption at 2 wk post-ISO-OV. In an effort to evaluate, in a more direct manner, cardiac contractility performance in ISO-OV-treated rats at 2 wk post-ISO-OV, studies in isolated hearts were performed.
performed under basal conditions and upon physiological β-adrenergic stimulation. Figure 2A shows pooled data of basal MPI and upon β-adrenergic stimulation, with increasing ISO concentrations (1–100 nM), for both control and ISO-OV hearts. We found no difference in MPI at basal conditions or at the lowest ISO concentration tested in ISO-OV hearts, compared with controls (P > 0.05). However, upon β-adrenergic stimulation with 10 and 100 nM ISO, ISO-OV hearts had a limited increase in contractility (38% increase above basal at stimulation with 10 and 100 nM ISO, ISO-OV hearts had a physiological range, MVO2 at the whole heart level (Fig. 2) performed under basal conditions and upon physiological β-adrenergic stimulation. Figure 2A shows pooled data of basal MPI and upon β-adrenergic stimulation, with increasing ISO concentrations (1–100 nM), for both control and ISO-OV hearts. We found no difference in MPI at basal conditions or at the lowest ISO concentration tested in ISO-OV hearts, compared with controls (P > 0.05). However, upon β-adrenergic stimulation with 10 and 100 nM ISO, ISO-OV hearts had a limited increase in contractility (38% increase above basal at the highest ISO concentration), whereas the control hearts had an increase of ~112% above basal, under similar experimental conditions (P < 0.05).

Similar to the blunted MPI response to ISO, within the physiological range, MVO2 at the whole heart level (Fig. 2B) showed no difference at basal conditions (4.39 ± 0.45 and 4.24 ± 0.51 μmol O2/min·g tissue, for control and ISO-OV hearts, respectively; P > 0.05), and ISO-OV hearts showed a significantly smaller increase at 100 nM ISO (9.21 ± 0.26 and 6.07 ± 0.08 μmol O2/min·g tissue, for control and ISO-OV hearts, respectively; P < 0.05).

Under normal conditions, the normalized MPI versus MVO2 relationship is linear, since any increase in contractility should usually be balanced with a proportional increase in ATP production due to oxidative phosphorylation (which is proportional to MVO2). Figure 2C shows this linear relation for control hearts upon increasing β-adrenergic stimulation. However, ISO-OV-treated hearts showed a limited response to ISO and presented a rightward shift, evidencing an apparent inefficient coupling between contraction and energy production, and suggesting that ISO-OV hearts required more respiratory activity to generate contractile force at any given ISO concentration.

In vitro mitochondria studies at 2 wk post-ISO-OV. Because the observed alterations in cardiac contractility and MVO2 could be due to deficient mitochondrial oxidative metabolism or alterations in cellular Ca2+ dynamics, we tested both possibilities, and first characterized mitochondrial function. In isolated mitochondria we measured NADH-linked or succinate-linked respiration velocity (respiratory chain complexes I and II, respectively). In ISO-OV mitochondria, we found a significant decrease in NADH-linked respiration velocity (65% and 59%, in state 3 and 4, respectively) and also in succinate-linked respiration (82% and 69%, respectively; Table 2).

Interestingly, in ISO-OV hearts we also found a significant decrease in mitochondrial yield (8.85 ± 0.47 and 7.05 ± 0.20 mg protein/g tissue, for control and ISO-OV, respectively; P < 0.05), suggesting increased mitochondrial fragility. Oxidative stress and Ca2+ overload are known to have detrimental effects on mitochondrial inner membrane integrity, since both are inducers of the mPTP configuration (7, 17, 24) and both are present in the acute phase of ISO-OV (13, 18). Figure 3A shows representative traces of Ca2+ uptake and retention capacity of control and ISO-OV mitochondria. Pooled data in Fig. 3B shows a significant decrease (~49%) in total Ca2+ tolerated by ISO-OV mitochondria. This decrease was abolished in the presence of Cyclosporine A, which is a well-known mPTP configuration blocker.

Because uncoupling between oxidative phosphorylation and respiratory chain activity in dysfunctional mitochondria is a predominant trigger of ROS production, by monitoring the aconitase activity, we indirectly determined that ISO-OV mitochondrial oxidative stress was enhanced (Fig. 3C).

Table 2. Mitochondrial respiration in ISO-OV rats at 2 wk post-insult

<table>
<thead>
<tr>
<th>Complex I</th>
<th>Control</th>
<th>ISO-OV</th>
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<tbody>
<tr>
<td>State 3</td>
<td>20.8 ± 0.7</td>
<td>13.6 ± 1.6*</td>
</tr>
<tr>
<td>State 4</td>
<td>9.9 ± 0.3</td>
<td>5.8 ± 0.5*</td>
</tr>
<tr>
<td>Respiratory control ratio</td>
<td>2.10 ± 0.01</td>
<td>2.4 ± 0.49</td>
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<tr>
<th>Complex II</th>
<th>Control</th>
<th>ISO-OV</th>
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<tr>
<td>State 3</td>
<td>32.2 ± 2.7</td>
<td>26.5 ± 0.06</td>
</tr>
<tr>
<td>State 4</td>
<td>16.6 ± 1.0</td>
<td>11.4 ± 0.4*</td>
</tr>
<tr>
<td>Respiratory control ratio</td>
<td>1.90 ± 0.06</td>
<td>2.3 ± 0.03*</td>
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Values are means ± SE; n = 4. Mitochondrial respiratory control was obtained by measuring the ratio between State 3 (ADP driven) respiration and State 4 (ADP depleted) respiration. Complex I respiration was obtained by using glutamate/malate as substrate. Complex II respiration was obtained by using succinate as substrate. *P < 0.05 vs. control.
**Intracellular Ca²⁺ handling at 2 wk post-ISO-OV.** Figure 4, A and B, shows representative steady state Ca²⁺ transients evoked by field stimulation for control and ISO-OV cells. Figure 4C shows pooled data for the time course of change for the Ca²⁺ transient peak amplitude upon physiological β-adrenergic stimulation (ISO, 100 nM) in both cell types. We found that, under basal conditions, peak Ca²⁺ transient amplitude was significantly lower in ISO-OV cells than that in control cells (8.3 ± 1.0 and 4.3 ± 0.5, for control and ISO-OV cells, respectively; \( P < 0.05 \)). After 10 min of continuous β-adrenergic stimulation, the Ca²⁺ transient amplitude increased only to 6.1 ± 1.4 in the ISO-OV cells, while the control group increased up to 14.0 ± 2.0 (\( P < 0.05 \)).

In order for cardiac cells to relax, released Ca²⁺ must be removed from the cytosol, and the SERCA and the sarcolemmal Na⁺/Ca²⁺ exchanger (NCX) perform this task. SERCA restores SR Ca²⁺ content, whereas the NCX removes Ca²⁺ to the extracellular space (8). Ca²⁺ transient time to 50% decay (T50%) provides an index of the combined activity of these Ca²⁺ removal systems; however, SERCA activity underlies 92% of Ca²⁺ removal (4). Figure 4D shows the Ca²⁺ transient T50% plotted under basal conditions (at 0 min) and as a function of time during continuous β-adrenergic stimulation. For ISO-OV cells, at basal conditions, T50% was 0.88 ± 0.11 s, whereas in control cells it was 0.37 ± 0.03 s (\( P < 0.05 \)). In both cases, there was an increase in cytosolic Ca²⁺ removal rate after 10 min of 100 nM ISO exposure; however, the difference between control and ISO-OV cells persisted (0.71 ± 0.10 and 0.22 ± 0.01 s, for control and ISO-OV cells, respectively; \( P < 0.05 \)).

Because peak Ca²⁺ transient amplitude depends on SR Ca²⁺ content, we estimated steady state SR Ca²⁺ content by the cytosolic peak of the caffeine-evoked Ca²⁺ release in both cell types. Figure 5, A and B, shows representative confocal images of caffeine-evoked SR Ca²⁺ release, in control and ISO-OV cells, respectively. Pooled data of peak caffeine-evoked Ca²⁺ release is shown in Fig. 5C. ISO-OV cells showed significantly lower SR Ca²⁺ content (7.8 ± 0.5 and 5.1 ± 0.4, for control and ISO-OV cells, respectively; \( P < 0.05 \)).

Because alterations in Ca²⁺ handling in other pathological settings are also reflected at the level of diastole, we investigated whether spontaneous Ca²⁺ sparks were altered in ISO-OV cells. Figure 6A shows typical Ca²⁺ sparks recordings for control (above) and ISO-OV cells (below), under basal conditions and upon 100 nM ISO exposure. Figure 6B shows pooled data for Ca²⁺ spark frequency plotted under basal conditions (0 min) and as a function of time during continuous β-adrenergic stimulation. We found that cells from the ISO-OV animal model had significantly higher Ca²⁺ spark frequency at basal conditions (0.61 ± 0.17 and 1.56 ± 0.31, for control and ISO-OV cells, respectively; \( P < 0.05 \)) and showed only a modest increase after 10 min of β-adrenergic stimulation (2.12 ± 0.34; \( P > 0.05 \)), in contrast with the significant increase in control cells (1.71 ± 0.26; \( P < 0.05 \)). Ca²⁺ spark frequency at 10 min of ISO exposure was not different between both cell types (\( P > 0.05 \)). Additionally, in ISO-OV cells we found a significant decrease in the mean Ca²⁺ spark amplitude, compared with control, in both basal and upon β-adrenergic stimulation (Fig. 6C). Ca²⁺ spark duration (full duration at half-maximal amplitude) was also increased; however, this difference was not significant at baseline and after 10 min of ISO perfusion (Fig. 6D). Finally, Ca²⁺ spark width (full width at half-maximal amplitude) did not show any difference in both cell types, in basal conditions and upon β-adrenergic stimulation (not shown).

**β-Adrenergic signaling pathway and expression of key Ca²⁺ handling proteins at 2 wk post-ISO-OV.** Increased Ca²⁺ spark frequency in ISO-OV cells, under basal conditions could be explained by an abnormally high β-adrenergic stimulation under basal conditions, which might affect the phosphorylated...
state of proteins as the RyR2, among others. To test this possibility, we assessed basal cAMP and PKA activity levels in both cell types and found those parameters to be unchanged in ISO-OV cells (Fig. 7).

Blunted Ca\(^{2+}\) transient with slow decay and low SR Ca\(^{2+}\) content could be due to decreased expression in SERCA or changes in its inhibitor PLB or decreased RyR2. Furthermore, under physiological conditions, catecholamines accelerate Ca\(^{2+}\) transient decay by increasing SERCA function due to PKA-dependent PLB phosphorylation (29); hence, a defective decrease in the SERCA-to-PLB expression ratio would also decrease the β-adrenergic SERCA Ca\(^{2+}\) removal responsiveness. We assessed SERCA, PLB, and RyR2 protein levels in both cell types; however, we did not find changes in expression of any of those proteins (Fig. 8).

**DISCUSSION**

Similar to previous reports, a single ISO-OV, at a concentration in the lower range tested by others (52, 57, 65), caused acute cardiac apex damage (Supplemental Videos), which might be partially due to a higher density of β-adrenergic receptors (AR) in the apex (52, 60, 61) and the higher proportion of β\(_2\) AR-linked to G\(_i\) than G\(_s\) proteins (38, 52). More importantly, we found after 2 wk of ISO-OV, significant in vivo systolic and diastolic dysfunction (Table 1). Nevertheless, systolic function returned to basal levels at 4 wk post-ISO-OV, while diastolic dysfunction worsened (Fig. 1). This is in agreement with original studies done by Teerlink et al. (57), in which animals treated with ISO doses <80 mg/kg did not progress, at several weeks follow-up, toward systolic heart failure, yet did develop a mild to moderate diastolic cardiomyopathy, which was ISO dose dependent (57). Therefore, rats treated with 67 mg/kg ISO appear to be an appropriate model of SIC, highlighting the importance of exploring the mechanisms of the transient systolic dysfunction, observed at 2 wk. In this regard, isolated hearts showed an impaired contractile-metabolic coupling during the higher metabolic demand imposed by physiological β-adrenergic stimulation (Fig. 2). Parallel in vitro studies showed altered mitochondrial oxidative metabolic state (Table 2), increased mitochondrial fragility (Fig. 3, A and B), and oxidative stress (Fig. 3C). Similar changes have been previously found in mitochondria from HF (28). In isolated cells we found dysfunctional systolic and diastolic Ca\(^{2+}\) handling, which was largely insensitive to β-adrenergic stimulation (Figs. 4 and 6); however, this was not correlated with SERCA, PLB, or RyR2 changes in expression (Fig. 8). As discussed below, energy synthesis impairment (12, 28, 58) and exacerbated ROS production (35), as a consequence of persistent mitochondrial dysfunction, might underlie many of the functional alterations in the cell, as well as in the whole heart at 2 wk post-ISO-OV.

The inefficient contractility-MVO\(_2\) coupling (Fig. 2C) led us to believe in the existence of underlying mitochondrial dysfunction. To assess this hypothesis we studied respiration in isolated ISO-OV mitochondria (Table 2) and found that ADP-stimulated respiration rate (state 3) was significantly decreased, suggesting that ISO-OV cells could have limited ability to produce ATP. Early studies revealed that the respiratory chain and Krebs cycle enzymes activities are decreased in human and experimental models of HF (23, 36). Moreover, in dog models of pacing-induced HF, the loss of coupling between the rate of electron transport and oxidative phosphorylation are gradually coincident with a reduction in ATP levels during the progression to HF (54, 59). These findings are in agreement with those reported during the acute phase of ISO-OV (13) and could explain the uncoupling between contractility and MVO\(_2\) reported here.

Decreased cardiac contractility could also be due to alterations in Ca\(^{2+}\) signaling. Therefore, in parallel experiments, we assessed systolic (Fig. 4) and diastolic Ca\(^{2+}\) (Fig. 6) handling in ventricular myocytes at 2 wk post-ISO-OV. We found that the electrically evoked Ca\(^{2+}\) transients were blunted and with a slower decay (Fig. 4) and largely insensitive to physiological β-adrenergic stimulation. Furthermore, spontaneous Ca\(^{2+}\) sparks in resting conditions occurred at higher frequency, albeit with smaller amplitude (Fig. 6), and they were unaffected by ISO (100 nM). Furthermore, basal SR Ca\(^{2+}\) content was lower (Fig. 5). All these changes in Ca\(^{2+}\) handling are characteristic of human and animal models of HF (9, 26, 31, 39, 41), and various molecular mechanisms have been proposed to explain them. The reduced Ca\(^{2+}\) transient amplitude could be due either to decreased SR Ca\(^{2+}\) content or a decrease in L-type Ca\(^{2+}\) channel current density (I\(_{Ca-L}\)). The general consensus is that there is only a modest or no change in I\(_{Ca-L}\),
even in the context of pathologies as profound as HF (49, 56). On the other hand, there is much more evidence associated with a state of SR Ca\(^{2+}\)/H\(^{+}\) depletion in pathologies such as ischemic and nonischemic HF (26, 26, 41, 51). Because RyR2 density was unchanged (Fig. 8), the lower intra SR Ca\(^{2+}\)/H\(^{+}\) content in ISO-OV cells shown in Fig. 5 could explain the smaller amplitude of Ca\(^{2+}\)/H\(^{+}\) transients and of Ca\(^{2+}\)/H\(^{+}\) sparks (Figs. 4 and 6); however, this does not explain the higher Ca\(^{2+}\) spark frequency (see below).

The lower SR Ca\(^{2+}\) content in ISO-OV cells, as well as the slow rate of Ca\(^{2+}\) transient decay (Figs. 4 and 5), could be explained by decreased SERCA expression, which is a fairly common finding in HF (2, 21, 39). However, our assessment of SERCA and PLB expression did not show any difference in protein levels in ISO-OV myocytes (Fig. 8). Nevertheless, it should also be noted that there is great heterogeneity in the studies of SERCA expression in human HF, as well as in animal models of HF (39). Various studies have shown that decreased SERCA activity is not necessarily accompanied by decreased protein expression (25, 32, 48, 64) and, as pointed out by Wold et al. (64), Ca\(^{2+}\) signaling alterations, at least in some animal models, may precede changes in protein expression (64). Given the role of \(\beta\)-adrenergic stimulation in enhancing SERCA function, which underlies ISO inotropic and lusitropic effects (8), we also sought to determine possible alterations in the \(\beta\)-adrenergic signaling pathway (60); however, we found no alterations in PKA basal function (Fig. 7B), and the cAMP levels were also within normal range (Fig. 7A). Unfortunately, we were unable to assess the density of \(\beta_1\)-adrenergic receptors. Nevertheless, based on our PKA and cAMP data, we suggest that \(\beta\)-adrenergic signaling is intact in ISO-OV hearts, and therefore could not underlie the lack of response to physiological ISO. To explain diminished SERCA capacity to reload the SR we should consider the consequences of mitochondrial dysfunction, particularly during high cardiac workloads. A reduction in the mitochondrial phosphorylation potential can lead to a reduced capacity for energy supply and affect ATPases involved in Ca\(^{2+}\) handling (12, 28, 58) and impair removal of the end products of ATP hydrolysis, all of which have been observed in the failing myocardium (23, 59).

SERCA is one of the most energy-demanding, and therefore, most sensitive cardiac enzyme to ATP depletion (28, 39, 58). Therefore, dysfunctional mitochondria, as assessed here (Table 2 and Fig. 3), as well as diminished glycolysis, as others have
reported in HF (12, 28), or decreased energy reserve via the creatine kinase reaction (58) may undermine cytosolic Ca\(^{2+}\) removal by SERCA, decrease contractile strength, and slow muscle relaxation, as reported here.

On the other hand, even though the SR Ca\(^{2+}\) content was lower in ISO-OV myocytes (Fig. 5), under basal conditions we found increased spontaneous Ca\(^{2+}\) sparks frequency (Fig. 6). Because the steady state SR Ca\(^{2+}\) content results from the balance between SERCA activity and SR "Ca\(^{2+}\) leak" (50), the latter mainly reflected as spontaneous Ca\(^{2+}\) sparks (Fig. 6), the increased SR Ca\(^{2+}\) leak might partially explain the lower SR Ca\(^{2+}\) content observed here. These findings are in agreement with other reports in HF and in other models, where diastolic Ca\(^{2+}\) release is increased (6, 15, 31, 51), and hyper-active RyR2 in resting conditions have been reported. It has been hypothesized that the abnormally high RyR2 activity might occur as a result of enhanced basal RyR2 phosphorylation, due either to PKA (31) or CaMKII (16, 62) or, more recently, by RyR2 oxidation (6, 66). Although we did not measure RyR2 phosphorylation or specific RyR2 oxidation in ISO-OV cells, because in basal conditions we did not find altered cAMP levels or PKA activity, we believe that PKA-dependent phosphorylation of the RyR2 might not be the cause of RyR2 hyperactivity. Additionally, β-adrenergic stimulation did not increase Ca\(^{2+}\) sparks in ISO-OV cells (Fig. 6), and others have argued that this is due to an already maximal level of RyR2 phosphorylation; nevertheless, we argue that instead this might be due to enhanced basal RyR2 oxidation. In this regard, RyR2 function is known to be upregulated by mitochondrial ROS-dependent oxidation of RyR2-thiol groups, in pathological conditions (6, 66), or even under normal conditions (aging) (15), and upon β-adrenergic stimulation (10). Because we found an apparent increase in mitochondrial oxidative stress, as inferred from the decrease in the aconitase activity (Fig. 3C), we propose that the RyR2 might be oxidized, and this could, at least partially, underlie the increased spontaneous SR Ca\(^{2+}\) release activity in ISO-OV cells under basal conditions (6, 10, 15, 66). Enhanced diastolic Ca\(^{2+}\) leak could not only contribute to the decreased SR Ca\(^{2+}\) content but also would increase the risk of delayed after depolarizations and triggered arrhythmias typical of several models of HF (1, 44, 45).

Furthermore, mitochondrial fragility, as we found here (Fig. 3, A and B), has been previously reported in ischemic injury or HF (20, 28, 43, 53). Other studies have shown that pharmacological and conditional inhibition of mPTP formation significantly improved cardiac function by reducing ischemic injury and myocardial infarct size in animal models and patients (20, 43). Therefore, our data would imply that mPTP opening, as consequence of mitochondrial dysfunction, is also involved in the pathological mechanism of post-acute catecholamine toxicity.

Finally, this study includes a thorough characterization of the ISO-OV model aimed to establish a link between mitochondrial dysfunction and intracellular Ca\(^{2+}\) mishandling with the transient cardiac dysfunction occurring at 2 wk after a single ISO-OV injection (67 mg/kg sc). This ISO dose was selected because it is well within the range (50–100 mg/kg sc) where acute cardiac damage is clearly induced (Supplemental Video S2), yet acute and long-term mortality is relatively low (13, 52, 57). Indeed at further follow-up, in line with what other authors have shown, systolic function apparently recovers at the expense of increased ventricular volumes and impaired ventricular relaxation (Fig. 1) (57).

In conclusion, this work has helped to get a better knowledge of the impact of catecholamine toxicity once the acute ischemic damage stopped progressing in the heart (57). Our findings showed that the contractile dysfunction at 2 wk was accompanied by altered mitochondrial functional state, intracellular Ca\(^{2+}\) mishandling, and decreased β-adrenergic responsiveness. Both contractile dysfunction as well as Ca\(^{2+}\) signaling may result as consequence of mitochondrial impairment, with decreased ATP synthesis capacity and increased ROS production. Although progression of cardiac dysfunction is a hallmark of HF (1, 30), cardiac dysfunction found in the setting of SiC is reversible once the catecholamine surge subsides (60); therefore, the ISO-OV model described here should be appropriate to study SiC (52).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES

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