Original Article

Cardioprotection Induced by Activation of GPER in Ovariectomized Rats With Pulmonary Hypertension

Allan K. N. Alencar, PhD,1 Guilherme C. Montes, PhD,1 Daniele G. Costa, PhD,1 Luiza V. P. Mendes, PhD,1,2 Ananssa M. S. Silva, PhD,1 Sabrina T. Martinez, PhD,3 Margarete M. Trachez, MD, PhD,1 Valéria do M. N. Cunha, PhD,1 Tadeu L. Montagnoli, B.Ch.E.,1 Aline G. M. Fraga, PhD,4 Hao Wang, MD, PhD,5 Leanne Groban, MD,5 Carlos A. M. Fraga, PhD,1 Roberto T. Sudo, MD, PhD,1 and Gisele Zapata-Sudo, MD, PhD1

1Programa de Pesquisa em Desenvolvimento de Fármacos, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Brazil. 2Departamento de Farmacologia, Universidade Estácio de Sá, Rio de Janeiro, Brazil. 3Departamento de Química, Instituto de Química, Campus do Valonguinho, Universidade Federal Fluminense, Niterói - RJ, Brazil. 4Faculdade de Farmácia da Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Ilha do Fundão Cidade Universitária, Brazil. 5Departments of Anesthesiology and Internal Medicine-Molecular Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina.

Address correspondence to: Gisele Zapata-Sudo, MD, PhD, Universidade Federal do Rio de Janeiro, Centro de Ciencias da Saude, Instituto de Ciencias Biomedicas, Bloco J, Sala 14, Rio de Janeiro, RJ 21941-590, Brazil. E-mail: gzsudo@oi.com.br

Received: August 4, 2017; Editorial Decision Date: March 15, 2018

Decision Editor: Rafael de Cabo, PhD

Abstract

Pulmonary hypertension (PH) is a disease of women (female-to-male ratio 4:1), and is associated with cardiac and skeletal muscle dysfunction. Herein, the activation of a new estrogen receptor (GPER) by the agonist G1 was evaluated in oophorectomized rats with monocrotaline (MCT)-induced PH. Depletion of estrogen was induced by bilateral oophorectomy (OVX) in Wistar rats. Experimental groups included SHAM or OVX rats that received a single intraperitoneal injection of MCT (60 mg/kg) for PH induction. Animals received s.c. injection of either vehicle or G1, a GPER agonist, (400 µg/kg/day) for 14 days after the onset of disease. Rats with PH exhibited exercise intolerance and cardiopulmonary alterations, including reduced pulmonary artery flow, biventricular remodeling, and left ventricular systolic and diastolic dysfunction. The magnitude of these PH-induced changes was significantly greater in OVX versus SHAM rats. G1 treatment reversed both cardiac and skeletal muscle functional aberrations caused by PH in OVX rats. G1 reversed PH-related cardiopulmonary dysfunction and exercise intolerance in female rats, a finding that may have important implications for the ongoing clinical evaluation of new drugs for the treatment of the disease in females after the loss of endogenous estrogens.

Keywords: Estrogen, Ovariectomy, Cardiopulmonary system, Right ventricle failure

Pulmonary hypertension (PH) is a heterogeneous group of diseases characterized by increased blood pressure in pulmonary arteries (PA), which complicates the majority of cardiovascular and respiratory conditions (1). The current classification of PH categorizes these multiple conditions into five groups according to their similar clinical presentation, pathologic findings, hemodynamics, and treatments (2). From this classification, pulmonary arterial hypertension (PAH; Group 1 PH) is the most devastating and progressive form of PH that has a multifactorial etiology and might result in right ventricle (RV) dysfunction, failure, and premature death (3–5).

PAH has been described as a disease of young and middle-aged women, with more recent registries showing the female-to-male ratios ranging from 1.4:1 to 4.1:1 (6–14). However, the age of most patients diagnosed with PAH has been increasing and it is not uncommon to see elderly patients with this cardiopulmonary disease (6,9,14–16). Compared with their younger counterparts, elderly patients with PAH have more exercise
intolerance, poorer responsiveness to PAH-targeted therapies, and reduced survival rates (16).

Estrogen (E₂) has a direct RV cardioprotective effect since women receiving postmenopausal E₂ replacement have reduced RV dysfunction (17). Thus, when female sex is accounted as a risk factor for PAH, it can be assumed that ovarian sex hormone actions are important predictors of outcomes in this class. Additionally, changes in the E₂ milieu caused by aging in postmenopausal women may influence PAH epidemiology and favor disease progression, signified by decompensated RV failure and increased symptom severity. Loss of ovarian estrogens in the context of aging, could explain, in part, the poorer cardiopulmonary prognostic found among elderly women when compared to younger women with PAH (16).

Despite PAH being more frequently observed in women, a personalized medicine approach is not yet considered. E₂ administration and inhibition of its metabolism have shown beneficial effects in animal models of PAH (18–24). Nevertheless, considering E₂ administration as a therapeutic strategy for women with PAH in the future would represent a challenge because of the “estrogen paradox” and the appearance of side effects consequent to the activation of classic E₂ receptors, such as cell proliferation on breast or reproductive tissue (25). Numerous preclinical studies have shown the cardioprotective potential of the recently discovered G-protein coupled estrogen receptor (GPER) in rodent models of left ventricle (LV) dysfunction and systemic hypertension (26–34). We recently reported that age- and estrogen-related alterations on the LV diastolic and structural profile of senescent, ovariectomized (OVX) female rats are attenuated by chronic activation of GPER (34). The selective stimulation of GPER in old female rats also attenuated the adverse effects of late-life estrogen loss on exercise intolerance and skeletal muscle fatigue (35). Furthermore, GPER activation showed salutary effects in male rats with monocrotaline (MCT)-induced PH and RV dysfunction (36). Whether GPER activation represents a promising therapeutic strategy among females with PAH, particularly after the loss of ovarian estrogens, remains speculative.

In this study, we examined the role of GPER in the cardiopulmonary system from adult female Wistar rats with MCT-induced PH, following a bilateral ovariectomy, in order to mimic the cardiovascular phenotype of estrogen-deficient women with PAH. We hypothesized that the selective agonist of GPER, G1 (37), would reverse cardiopulmonary and skeletal muscle dysfunction from chemically-induced PH female rats with low levels of endogenous E₂.

**Methods**

**Drugs and Reagents**

G1 was dissolved in peanut oil (Sigma-Aldrich, St. Louis, MO). MCT was synthesized by the Laboratório de Química at the Universidade Federal Fluminense (UFF), Brazil. Before use, MCT was dissolved in 1 N HCl, neutralized with 0.5 N NaOH, and diluted with phosphate-buffered saline. Phenylephrine (Phe) and ACh from Sigma-Aldrich were dissolved in distilled water. Primary antibodies against sarco/endoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a), phospholamban (PLB), phosphorylated phospholamban (p-PLB), and glyceraldehyde 3-phosphate dehydrogenase were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was obtained from Abcam Corp. (Cambridge, MA).

**Animals and Experimental Design**

All animal procedures were approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro (n° 043/15).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Experimental timeline in days for the monocrotaline (MCT)-induced pulmonary hypertension protocol. Thirty days after acclimation period, ovariectomy or SHAM surgeries were performed in 9-week-old female rats. Following 1 week of recovery, MCT was injected at a dose of 60 mg kg⁻¹ i.p. on Day 1. Fourteen days (washout period) after MCT injection, OVX rats were subcutaneously treated with G1 (400 µg/kg/day) for 14 days. On Days 1, 14, and 28, echocardiographic examinations were performed to evaluate cardiopulmonary changes in function and structure. On Day 29, right ventricle (RV) catheterization was performed to measure RV systolic pressure (RVSP). Following this measurement, the animals were euthanized, the hearts were removed and weighed, and the RV was dissected. Pulmonary arteries (PAs) were rapidly removed for the vascular reactivity study. Representative images of the PA outflow profile are presented for (B) Day 1, (C) Day 14, and (D) Day 28 after MCT injection in a SHAM and OVX animals treated with MCT and OVX treated with MCT and G1. Note the progressive change in the shape of the PA waveform from Day 14 to Day 28 after MCT injection, which confirmed the development of PH. OVX = Ovariectomy; SHAM = Sham treatment. G1 reversed the mid-systolic notch.

Thirty-three female Wistar rats (9 weeks of age; 220–300 g, n = 33) were housed at 20 ± 3°C under a 12-hour light/12-hour dark cycle with free access to food and water. Figure 1A shows the experimental timeline used to characterize the effects of MCT, estrogen status and GPER activation on cardiac structure/function, PA, and RV hemodynamics. Following an acclimation period of 30 days, rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.) and underwent either a bilateral ovariectomy (OVX; n = 18) or their gonads were left intact (SHAM; n = 15), as previously published (38). After a recovery time of 7 days, SHAM rats were divided into two groups: control and MCT + vehicle (peanut oil), and OVX rats were divided into three: control, MCT + vehicle (peanut oil), and MCT + G1. To induce PH, rats in MCT groups received single intraperitoneal (i.p.) injections of MCT at 60 mg/kg body weight 1 week following OVX/sham surgery (34,39,40). Rats in control groups received single i.p. injections of sterile saline at volumes equivalent to the MCT dosing.

As the basal production of ovarian estrogens are presumed to be maintained in SHAM animals in the presence and absence of PH, we did not study the effects of G1 in these rats since the pharmacological profile of GPER activation could be additive or even confounded by...
endogenous E2. Accordingly, 14 days after MCT or saline administration (washout period), both SHAM and OVX rats were dosed once daily for 2 weeks with 0.1 mL of peanut oil (control and MCT + vehicle groups), and G1 was administrated at a dose of 400 µg/kg body weight, subcutaneously (MCT + G1 group), only in OVX rats challenged with MCT. Rats were weighed daily, and the dosages of G1 were adjusted appropriately. This dose of G1 was chosen based on our recently published findings of its beneficial effects in male rats with PH, and its lack of vasodilator activity within the systemic circulation (36).

Exercise Test Protocol
The time to exhaustion was calculated during a standardized exercise protocol that was performed using a motorized-treadmill (EP-131, Insight, São Paulo, Brazil) (35). Briefly, rats were adapted to the rodent treadmill by walking at a speed of 20 cm/s, 10 minutes/d, for 1 week. Each exercise test was performed after at least 1 day of rest. The protocol for exercise performance evaluation consisted of 3 minutes at 12 m/min, with 1.2 m/min increases in speed every 3 minutes until the rats reached exhaustion. Time to exhaustion (in minutes) was determined when the rats stopped running.

Echocardiography
Cardiac function was assessed by a high-resolution ultrasound imaging system equipped with a RMV-710B transducer (25 MHz) (Vevo 770, Visualsonics, Toronto, Canada). Animals were kept at room temperature and were anesthetized with a 2% isoflurane/oxygen mixture. Short- and long-axis B-dimensional parasternal views of RV and LV were acquired to obtain their areas. Mitral inflow and tissue Doppler measurements of maximum early transmural filling velocity (E) and early mitral annular velocity (e'), respectively, were used to calculate the E/e' ratio to evaluate LV diastolic function. RV wall thickness was determined in M-mode. Doppler from the PA was applied to obtain the pulmonary artery acceleration time (PAAT).

Hemodynamic and RV Hypertrophy Measurements
After the final treadmill test (Day 29 of protocol), rats were anesthetized with ketamine (80 mg/kg, 2 i.p.) and xylazine (15 mg/kg, i.p.). Anesthesia depth was verified by pinching the animal’s paw with forceps. A polyethylene catheter, connected to a PowerLab pressure transducer (ADInstruments, Sydney, Australia), was inserted into the right carotid artery to measure the mean arterial blood pressure (MAP). Subsequently, the catheter was introduced into the LV to record intraventricular pressure. Another polyethylene catheter was inserted into the right jugular vein to measure the right-ventricle systolic pressure (RVSP). Immediately after the hemodynamic measurements were completed and deep anesthesia was confirmed, the animals were killed via exsanguination by cardiac puncture. All animals were examined for the presence or absence of ovaries and uteri were dissected and weighed. Uterus weights were used as a sign of a successful surgical procedure, as the gonadal removal would reduce the E2 uterotrophic effect (41).

Evaluation of PA Endothelial Function
After euthanasia, PAs from all experimental groups were collected. Ex vivo endothelial function using ACh was determined using our recently published work (36).

Membrane Preparations and Western Blot Analysis
Equal amounts of protein (20–60 µg) were separated by a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane using a semi-dry system (Bio-Rad, USA). Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20 and incubated with primary antibodies against SERCA2a, PLB, p-PLB, and glyceraldehyde 3-phosphate dehydrogenase (loading protein) (36). Signals were visualized by using a Super Signal West Pico Chemiluminescence Kit (Pierce, Rockford, IL). The membranes were exposed to radiographic films and protein band density was measured by using Scion Image Alpha 4.0.3.2 (Scion Corp., USA).

Histological Studies
RV samples were fixed by immersion in zinc formalin and embedded in paraffin. Tissues were sectioned (4 µm for lungs, 7 µm for RVs), stained (picrosirius red) and analyzed in a blinded fashion, as published recently (36).

Data Analysis
Data analysis was performed for all endpoints, and one-way analysis of variance was used to determine the significance of differences among groups. Significance of interactions between groups was determined by Tukey post-hoc tests. Pearson correlation was used to test for a relationship between PAAT and ACh efficacy. Differences between SHAM and OVX female rats were compared using unpaired Student’s t test. Differences for all tests were considered significant when the p value was less than .05. Analyses were performed using GraphPad Prism, version 6 (GraphPad, San Diego, CA).

Results
Animal Characteristics
Table 1 shows the physical characteristics of experimental groups. Final body weights did not change among SHAM rats. Similar to our previous report (43), OVX control rats exhibited a significant increase in body weight compared to its SHAM control counterpart. Administration of MCT at a dose of 60 mg/kg inhibited OVX-related increases in body weight, regardless of G1 treatment (Table 1). As the body weight was different among our experimental groups, we used tibial length to calculate indices of cardiac hypertrophy. In both SHAM and OVX rats, the MCT challenge produced significant increases in heart weight-to-tibial length and RV-to-LV + septal weight ratios, compared with respective controls. Importantly, the magnitude of cardiac hypertrophy was significantly greater in OVX- versus SHAM-MCT-challenged rats, and this OVX-associated remodeling effect was suppressed by chronic GPER activation via daily subcutaneous administration of G1 (Table 1). Uterine weight was significantly lower in the MCT-challenged SHAM rats compared to their vehicle (saline)-treated SHAM counterparts which might reflect an MCT-induced toxicity in ovaries. Additionally, uterine weights were lower in all the OVX regardless of G1 treatment, confirming both the efficacy of gonadectomy and the lack of a G1-mediated uterotrophic effect (44).
G1 Treatment Reversed PA Dysfunction and Reduced RV Overload in MCT-treated Female Rats

Doppler echocardiography was used to image the PA outflow waveform profile (Figure 2A). PH establishment was confirmed by a change in the shape of the PA waveform after MCT injection, as described previously (36). MCT-induced modifications in the PA flow pattern were greater in OVX rats when compared to SHAM rats, as depicted by the lower PAAT (Figure 2B) in MCT-challenged OVX versus MCT-challenged SHAM rats. Figure 2D additionally shows that ACh-induced PA relaxation correlates with PAAT. As expected, ex-vivo studies performed 29 days after the MCT challenge show reduced ACh efficacy in SHAM groups, 28 days after MCT injection. (C) Pulmonary artery acceleration time. (D) Maximal effect of ACh in isolated pulmonary arteries from experimental groups. (E) Linear regression between pulmonary artery acceleration time and ACh efficacy. Data represent the mean ± SEM (n = 5–7 rats per group). *p < .05 compared with corresponding control rats; †p < .05 compared with corresponding monocrotaline rats; ‡p < .05 compared with corresponding SHAM rats.

to MCT-challenged SHAM rats. OVX rats receiving two weeks of chronic G1 showed significant attenuations in MCT-associated RV area enlargements, and in MCT-associated LV area reductions when compared to vehicle-treated, MCT-induced PH OVX rats (Figure 3B and C, respectively).

GPER Activation by G1 Reversed RV Wall Hypertrophy and Reduced Collagen Deposition in MCT-treated Female Rats

Figure 4 shows M-mode echocardiographic images and picrosirius red staining of RVs from all experimental groups (Figure 4A and B, respectively). The higher degree of RV overload in MCT-challenged rats led to marked RV hypertrophy, as demonstrated by the significant increase in RV free wall thickness in both SHAM and OVX rats treated with MCT versus saline (control)-treated counterparts (Figure 4C). Correspondingly, RV collagen content was significantly higher in MCT-OVX rats compared to MCT-SHAM rats (Figure 4D). Interestingly, collagen deposition in the RV free wall, albeit modest, was also higher in OVX versus SHAM-unstressed rats. The increased collagen deposition might explain, in part, the greater RV free wall thickness observed in hearts from OVX- versus SHAM-MCT-subjected rats (Figure 4A). Important, G1 treatment reversed all of these deleterious effects of MCT-induced RV remodeling in rats lacking of ovarian estrogens (Figure 4C and D, respectively).
The effects of MCT injection and G1 administration on systemic and intracardiac pressures and LV systolic and diastolic function in OVX-MCT rats when compared to their vehicle-treated counterparts. Additionally, myocardial relaxation, as determined by tissue Doppler-derived early mitral annular velocity (e') was reduced in MCT-injected rats, compared to corresponding control rats, with a greater MCT effect being observed in those rats without ovaries (Table 2). Furthermore, Doppler-derived filling pressure, or the ratio of early filling-to-mitral annular descent (E/e'), was higher in both SHAM and OVX rats with PH compared to their corresponding control rats; with a greater MCT effect being observed in those rats without ovaries (Table 2). This lowering of systemic pressure might be a reflection of the reduced LV ejection fraction in OVX rats treated with MCT. Even though LV ejection fraction from MCT-challenged-SHAM rats showed a relative reduction compared to controls, it was essentially normal as it remained above 50% (Table 2). Treatment with G1 in OVX-MCT rats increased both systemic mean arterial and LV systolic pressures and augmented LV ejection fraction when compared to vehicle-treated OVX-MCT rats. Additionally, myocardial relaxation, as determined by tissue Doppler-derived early mitral annular velocity (e') was reduced in MCT-injected rats, compared to corresponding control rats, with a greater MCT effect being observed in OVX female rats (Table 2). Furthermore, Doppler-derived filling pressure, or the ratio of early filling-to-mitral annular descent (E/e'), was higher in both SHAM and OVX rats with PH compared to their respective controls. As expected, among rats with PH, E/e' was highest in those rats without ovaries (Table 2). Treatment with G1, at a dose of 400 µg/kg/day, reversed these impairments in LV diastolic function in OVX-MCT rats when compared to their vehicle-treated counterparts (Tables 2 and 3). Heart rate was significantly increased after MCT injection in SHAM rats. However, OVX rats with MCT-induced PH had a significant reduction in these hemodynamic parameters when compared to respective saline-injected controls (Table 3). This lowering of systemic pressure might be a reflection of the reduced LV ejection fraction in OVX rats treated with MCT. Even though LV ejection fraction from MCT-challenged-SHAM rats showed a relative reduction compared to controls, it was essentially normal as it remained above 50% (Table 2). Treatment with G1 in OVX-MCT rats increased both systemic mean arterial and LV systolic pressures and augmented LV ejection fraction when compared to vehicle-treated OVX-MCT rats. Additionally, myocardial relaxation, as determined by tissue Doppler-derived early mitral annular velocity (e') was reduced in MCT-injected rats, compared to corresponding control rats, with a greater MCT effect being observed in OVX female rats (Table 2). Furthermore, Doppler-derived filling pressure, or the ratio of early filling-to-mitral annular descent (E/e'), was higher in both SHAM and OVX rats with PH compared to their respective controls. As expected, among rats with PH, E/e' was highest in those rats without ovaries (Table 2). Treatment with G1, at a dose of 400 µg/kg/day, reversed these impairments in LV diastolic function in OVX-MCT rats when compared to their vehicle-treated counterparts (Tables 2 and 3). Heart rate was significantly increased in OVX animals treated with MCT in both echocardiographic

### Table 2. Cardiac Function

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCT + Vehicle</th>
<th>MCT + G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV ejection fraction (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>69.2 ± 5.2</td>
<td>59.4 ± 3.5*</td>
<td>59.7 ± 3.9</td>
</tr>
<tr>
<td>OVX</td>
<td>76.3 ± 2.8</td>
<td>26.2 ± 4.1*</td>
<td>621.7 ± 17.8</td>
</tr>
<tr>
<td>E (mm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>795.4 ± 22.7</td>
<td>727 ± 18.4</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>605.3 ± 4.0</td>
<td>608.9 ± 22.3</td>
<td></td>
</tr>
<tr>
<td>e' (mm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>82.7 ± 3.1</td>
<td>59.9 ± 5.5*</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>64.4 ± 2.1</td>
<td>29.9 ± 1.5*</td>
<td></td>
</tr>
<tr>
<td>E/e'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>9.6 ± 0.5</td>
<td>12.4 ± 0.9*</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>9.5 ± 0.6</td>
<td>22.0 ± 2.2*</td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>298.5 ± 4.0</td>
<td>321.2 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>302.3 ± 3.1</td>
<td>348.8 ± 6.7*</td>
<td></td>
</tr>
<tr>
<td>RSV (mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>0.23 ± 0.001</td>
<td>0.15 ± 0.001*</td>
<td>0.19 ± 0.001*</td>
</tr>
<tr>
<td>OVX</td>
<td>0.22 ± 0.01</td>
<td>0.05 ± 0.001*</td>
<td></td>
</tr>
<tr>
<td>RVEDV (mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>0.50 ± 0.01</td>
<td>0.66 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>0.46 ± 0.01</td>
<td>0.86 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>RVESV (mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>0.26 ± 0.01</td>
<td>0.50 ± 0.02*</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>0.23 ± 0.01</td>
<td>0.80 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>RVFS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>47.7 ± 1.6</td>
<td>24.0 ± 1.1*</td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>49.1 ± 2.6</td>
<td>6.6 ± 0.5*</td>
<td>45.4 ± 2.1*</td>
</tr>
</tbody>
</table>

Note: Each value represents the mean ± SEM (n = 5–7 rats per group; SHAM + vehicle, n = 7; SHAM + MCT + vehicle, n = 7; OVX + vehicle, n = 5; OVX + MCT + vehicle, n = 6; OVX + MCT + G1, n = 7). e' = Early mitral annular velocity; E = Maximum early transmural filling velocity; E/e' = Early transmural filling velocity-to-mitral annular velocity ratio; HR = Heart rate; LV = Left ventricle; MCT = Monocrotaline; OVX = Ovariectomy; RV = Right ventricle; RSV = Right ventricular stroke volume; RVEDV = Right ventricular end-diastolic volume; RVESV = Right ventricular end-systolic volume; RVFS = Right ventricular fractional shortening; SHAM = Ovary intact.

*p < .05 compared with corresponding control rats; †p < .05 compared with corresponding monocrotaline rats; ‡p < .05 compared with corresponding SHAM rats.

**Effects of G1 on RV Function and Intracellular Calcium Handling Proteins Expression**

**Figure 5A** shows the RV cardiac output from all the experimental groups. All rats treated with MCT had a significant reduction in RV cardiac output when compared to their saline-treated counterparts, and this effect was most pronounced in the OVX group (Figure 5A). Western blot analyses showed increased in RV SERCA2a expression and decreases in p-PLB-to-PLB ratio in MCT-injected rats versus control rats and this unfavorable calcium regulatory protein expression profile was even more pronounced in RV tissue from OVX rats challenged by MCT (Figure 5B and C, respectively). OVX rats with PH receiving chronic G1 showed higher RV cardiac outputs (Figure 5A), and normalization of SERCA2a expression (Figure 5B) and p-PLB-to-PLB ratios (Figure 5C) when compared to their corresponding vehicle-treated, OVX counterparts.

**G1 Treatment Normalized Hemodynamics and LV Dysfunction in MCT-treated Female Rats**

The effects of MCT injection and G1 administration on systemic and intracardiac pressures and LV systolic and diastolic function from SHAM and OVX female rats are summarized in Tables 2 and 3. Systemic mean arterial and LV systolic pressures did not change after MCT injection in SHAM rats. However, OVX rats with MCT-induced PH had a significant reduction in these hemodynamic parameters when compared to respective saline-injected controls (Table 3). This lowering of systemic pressure might be a reflection of the reduced LV ejection fraction in OVX rats treated with MCT. Even though LV ejection fraction from MCT-challenged-SHAM rats showed a relative reduction compared to controls, it was essentially normal as it remained above 50% (Table 2). Treatment with G1 in OVX-MCT rats increased both systemic mean arterial and LV systolic pressures and augmented LV ejection fraction when compared to vehicle-treated OVX-MCT rats. Additionally, myocardial relaxation, as determined by tissue Doppler-derived early mitral annular velocity (e') was reduced in MCT-injected rats, compared to corresponding control rats, with a greater MCT effect being observed in OVX female rats (Table 2). Furthermore, Doppler-derived filling pressure, or the ratio of early filling-to-mitral annular descent (E/e'), was higher in both SHAM and OVX rats with PH compared to their respective controls. As expected, among rats with PH, E/e' was highest in those rats without ovaries (Table 2). Treatment with G1, at a dose of 400 µg/kg/day, reversed these impairments in LV diastolic function in OVX-MCT rats when compared to their vehicle-treated counterparts (Tables 2 and 3). Heart rate was significantly increased in OVX animals treated with MCT in both echocardiographic
G1 Treatment Normalized Skeletal Muscle Changes in Female Rats With MCT-induced PH

Rats from all groups were submitted to a treadmill test before, 14 days after, and 28 days after MCT injection (Figure 1). The time to exhaustion was similar between the groups before MCT administration. Fourteen days after the pharmacologic induction of PH, the time to exhaustion was significantly reduced in both SHAM and OVX MCT-injected rats compared to respective controls (data not shown). By 28 days after PH induction, exercise tolerance was further reduced in all animals with MCT-induced PH (Figure 6A). Interestingly, saline-treated OVX group showed a significant reduction in treadmill performance compared to sham controls (Figure 6A). By the end of the protocol, MCT + G1 rats showed a longer time to exhaustion compared to MCT + vehicle OVX rats. Figure 6B shows selected trains of contractions at stimulation times of 0–60 minutes. Soleus maximal force peak was significantly reduced in both SHAM and OVX rats treated with MCT.
from 14 to 28 days, with a prominent effect being observed in OVX rats. Two weeks of chronic G1 administration in OVX rats with PH led to beneficial increases in PAAT, which may be explained, in part, by GPER’s antiremodeling effects on injured vascular tissue, presumably through its downgrading influences on glycosaminoglycan content and reactive oxygen species production (31). Furthermore, as G1 administration enhanced the ACh response in PA from OVX rats with PH, we suspect that GPER activation reduced PA wall stress by normalizing endothelial function and nitric oxide bioavailability, which is a potent vasodilator with anti-proliferative effects in pulmonary vessel cells (39). The normalization of PA flow by G1 also preserved the RV-PA coupling and the maintenance of RV hemodynamics and pressure–function relationships, as shown by the normal values of RV systolic pressure in MCT-challenged OVX rats treated with G1.

Persistent RV overload is the first contributor to RV myocardial remodeling, hypertrophy, failure, and premature death in patients with PH (45). In this study, MCT-induced PH in both SHAM and OVX rats led to RV fibrosis and hypertrophy by the end of protocol, and the intensity of this adaptive/maladaptive response was exacerbated in those rats deficient in ovarian estrogens. Treatment with G1 reduced RV collagen deposition, and hypertrophy with a subsequent preservation of RV and LV function, as depicted by the normal values of RV cardiac output, LVEF, and LV Doppler indices of diastolic function.

The global heart dysfunction and failure in patients with advanced PH is related to the higher degree of RV hypertrophy. A more spherical-shaped RV results in abnormal septal function that also impairs LV performance and structure (46). This corroborates with our experimental data and reinforces the cardioprotective potential of endogenous estrogens, that is, LV function and structure in PH-induced SHAM rats was essentially preserved when compared to OVX rats subjected to the same PH-producing challenge. The lower LV systolic and diastolic functional profile of PH-OVX rats was also reflected in systemic circulatory aberrations, as depicted by marked reductions in mean arterial pressure, another clinical sign of PH severity leading to heart failure. Moreover, the increased heart rate in OVX females with PH may be a compensatory mechanism of the autonomic nervous system to increase the reduced cardiac output and systemic pressures. Interestingly, OVX rats treated with G1 did not exhibit these changes in heart rate or systemic hemodynamics, likely due to direct actions of GPER on the heart. These data give an additional support to our previous work that shows the cardioprotective potential of G1 in hearts from male Wistar rats with MCT-induced PH (36), independently of sex influences on its expression and activity.

In addition to its beneficial actions on vascular endothelium and smooth muscle (47), the cardioprotective potential of G1 in hearts of PH female rats likely involves actions at both the cardiofibroblast and cardiomyocyte level. First, the in vivo activation of GPER by G1 attenuated the adverse effects of salt and/or estrogen loss on LV interstitial collagen deposition and myocyte hypertrophy in the female hypertensive, mRen2.Lewis rat (28,33). Extending this concept, Wang et al. (29) showed that in vitro GPER is expressed in cardiofibroblasts and that its activation is capable of limiting cell proliferation, probably through diminishing the effects of cell cycle proteins. Second, cardiomyocyte-specific deletion of GPER led to adverse LV remodeling and systolic and diastolic function in both male and female mice compared to their GPER-intact, wild-type counterparts (48). Third, GPER’s advantageous effects on the expression and activities of SERCA2a and PLB suggest that its beneficial

---

**Figure 6.** Effects of MCT injection on the exercise performance. Skeletal muscle function and protein expression over 28 days of protocol and subcutaneous treatment with vehicle or G1 (400 µg/kg/day) from Day 14 to 28 of protocol. (A) Treadmill time to exhaustion. (B) Soleus maximal force peak measured by the in vitro fatigue protocol. (C) Western blot analyses of SERCA2a (114 kDa), p-PLB (20 kDa), PLB (20 kDa), and GAPDH (37 kDa) expression in soleus muscle from MCT-challenged SHAM and OVX rats, respectively. GAPDH was used for normalization. (D) Quantification of SERCA2a expression. (E) Relative expression ratio of p-PLB to PLB. Each column and bar represent the mean ± SEM (n = 5–7 rats per group). *p < .05 compared with corresponding control rats; #p < .05 compared with corresponding monochrotalone rats; †p < .05 compared with corresponding SHAM rats. GAPDH = Glycerinaldehyde 3-phosphate dehydrogenase; MCT = Monocrotaline; OVX = Ovariectomy; PLB = Phospholamban; p-PLB = Phosphorylated phospholamban; RV = Right ventricle; SERCA2a = Sarco/endoplasmic reticulum Ca²⁺-ATPase 2a; SHAM = Ovary intact.

**Discussion**

In addition to our recently published work which addresses the salutary effects of G1 in the cardiopulmonary system of male rats with PH (36), we now show, for the first time, that selective activation of GPER by G1 in OVX rats subjected to pharmacologic induction of PH by MCT reverses or attenuates cardiopulmonary and skeletal muscle dysfunction when compared to corresponding OVX-MCT-challenged rats treated with vehicle. Furthermore, we have corroborated findings from Nishida et al. in that female rats devoid of ovarian estrogens exhibit more pronounced cardiopulmonary and skeletal muscle aberrations with PH than gonadal intact counterparts exposed to the same PH-inducing challenge (38).

Hypertrophy of small pulmonary vessels, along with exacerbated pulmonary vascular smooth muscle cell (PVSMC) contraction, work together to increase pulmonary vascular resistance, which, in turn, leads to increases in RV and PA pressures through the development of PH (1,44). In our data, we show that MCT-induction of PH impairs PA flow, as depicted by a progressive reduction in PAAT compared to their corresponding saline-injected controls. Treatment with G1 partially reversed MCT-related changes in soleus peak contraction in OVX rats with PH (Figure 6B). SERCA2a and p-PLB-to-PLB ratio expression in soleus muscle did not change among SHAM rats, but were significantly reduced in OVX animals treated with MCT. Treatment with G1 reversed these changes in skeletal muscle expression of intracellular calcium handling proteins (Figure 6C and D, respectively).
actions at the myocyte level also involve preservation of intracellular calcium regulation at times of physiologic (eg, aging, estrogen loss) and pharmacologic stress (eg, MCT-induced PH) (34). Indeed, many of our findings in the present study, including attenuated RV fibrosis, recovered LV function, improved calcium regulatory protein expression, and enriched ACh efficacy of PA segments following chronic G1 treatment, corroborate these distinct cardiopulmonary benefits of this noncanonical estrogen receptor.

GPER is expressed in rat skeletal muscle cells and its activation may offset the adverse effects of estrogen loss. Eight weeks of chronic G1 administration (100 μg/kg/day) in normotensive, old-aged female rats limited the exercise intolerance incited by late-life OVX, in part by regulating heat shock proteins in skeletal muscle (35). In the present study, we also show that GPER activation by G1 can attenuate PH-induced exercise intolerance, presumably by indirectly improving cardiopulmonary dynamics and directly increasing the force development of skeletal muscle. In addition to GPER’s modulation of heat shock proteins in skeletal myocytes, we now show that increases in soleus muscle contractility after G1 treatment may occur through the normalization of the calcium handling proteins, SERCA2a and phospholamban. Certainly, to thoroughly and specifically determine the roles of GPER in skeletal muscle during physiologic (eg, aging, estrogen loss) and pathologic perturbations (eg, PH, heart failure), the development of more targeted approaches, such as the inducible skeletal muscle specific GPER knockout mouse, will be needed. Nonetheless, the recovery of skeletal muscle function and exercise capacity by G1 in the PH-induced OVX Wistar rat provides insight into the capabilities of this new estrogen receptor as a pharmacological target for future strategies in the treatment of clinical PH, particularly among women with estrogen deficiency and advanced RV dysfunction and failure.

Clinical Relevance
Right ventricular performance and structure are the main predictors of survival in patients with PAH. Because sexual hormone levels are significantly correlated to a preserved RV function in both women and men (17), in this study, we have provided corroborating data showing that targeting the activation of a noncanonical estrogen receptor, specifically GPER, might represent an advantageous strategy to treat those patients with PH-induced RV dysfunction who are recalcitrant to present-day pharmacologic regimens, with a special attention to estrogen deficient women. We further propose that selective activation of GPER could be used to attenuate the main symptoms of PH and its subforms, that is, fatigue and exercise intolerance, thus improving prognosis and quality of life. In addition, developing drugs that specifically activate GPER, as opposed to using estradiol as an agonist, could eliminate many of the adverse genomic actions that have been associated with hormone replacement therapy.

Funding
This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ); Instituto Nacional de Ciência e Tecnologia de Fármacos e Medicamentos (INCT-INOFAR, Proc. 465.249/2014-0); and the National Institute on Aging (AG-033727 to L.G.) at the National Institutes of Health.

Conflict of Interest
None reported.

References
18. Renda TC, Kanagy NL, Walker BR. Estradiol-induced attenuation of pulmonary hypertension is not associated with altered eNOS expression.


