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No Effect of Endoperoxide 4 or Thromboxane A₂ Receptor Blockade on Static Mechanoreflex Activation in Rats with Heart Failure

Alec L. E. Butenas¹, Korynne S. Rollins¹, Jacob E. Matney¹, Auni C. Williams¹, Talyn E. Kleweno¹, Shannon K. Parr¹, Stephen T. Hammond¹, Carl J. Ade¹, Karen S. Hageman², Timothy I. Musch^{1,2}, Steven W. Copp¹

¹Department of Kinesiology, Kansas State University, Manhattan, KS

²Department of Anatomy and Physiology, Kansas State University, Manhattan, KS

Abstract

We investigated the role of cyclooxygenase metabolite-associated endoperoxide 4 receptors (EP4-R) and thromboxane A₂ receptors (TxA₂-R) on thin fiber muscle afferents in the chronic mechanoreflex sensitization in rats with myocardial infarction-induced heart failure with reduced ejection fraction (HF-rEF). We hypothesized that injection of either the EP4-R antagonist L-161,982 (1 µg) or the TxA₂-R antagonist daltroban (80 µg) into the arterial supply of the hindlimb would reduce the increase in blood pressure and renal sympathetic nerve activity (RSNA) evoked in response to 30 s of static hindlimb skeletal muscle stretch (a model of isolated mechanoreflex activation) in decerebrate, unanesthetized HF-rEF rats but not sham operated control rats (SHAM). Ejection fraction was significantly reduced in HF-rEF (45±11%) compared to SHAM (83±6%; $P<0.01$) rats. In SHAM and HF-rEF rats, we found that the EP4-R antagonist had no effect on the peak increase in mean arterial pressure (peak MAP SHAM $n=6$, pre: 15±7, post: 15±9, $P=0.99$; HF-rEF $n=9$, pre: 30±11, post: 32±15mmHg, $P=0.84$) or peak increase in RSNA (peak RSNA SHAM pre: 33±14, post: 47±31%, $P=0.94$; HF-rEF, pre: 109±47, post: 139±150%, $P=0.76$) response to stretch. Similarly, in SHAM and HF-rEF rats we found that the TxA₂-R antagonist had no effect on the peak MAP (SHAM $n=7$, pre: 13±7, post: 19±14, $P=0.15$; HF-rEF $n=14$, pre: 24±13, post: 21±13mmHg, $P=0.47$) or peak RSNA (SHAM pre: 52±43, post: 57±67%, $P=0.94$; HF-rEF, pre: 108±93, post: 88±72%, $P=0.30$) response to stretch. The data do not support a role for EP4-Rs or TxA₂-Rs in the chronic mechanoreflex sensitization in HF-rEF.

Corresponding author: Steven W. Copp, Kansas State University, Department of Kinesiology, 144 Justin Hall, Manhattan, KS 66506, Telephone: 785-532-1096, scopp@ksu.edu.

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Keywords

Heart failure reduced ejection fraction; COX-2; Exercise Pressor Reflex; Sympatho-excitation; Exercise Tolerance

1. Introduction

Approximately 6.2 million Americans have heart failure (Benjamin *et al.*, 2019), roughly half of which have heart failure with reduced ejection fraction (HF-rEF). Patients with HF-rEF exhibit exaggerated sympathetic nervous system activity (*i.e.* sympatho-excitation) at rest and during low intensity exercise (Leimbach *et al.*, 1986; Barretto *et al.*, 2009), which may contribute to the exercise intolerance commonly observed in this patient population (Haykowsky *et al.*, 2011; Shimiaie *et al.*, 2015). Moreover, this sympatho-excitation may place the individual at an increased risk of an ischemic event (*i.e.* heart attack or stroke). Thus, furthering our understanding of the mechanisms behind this sympatho-excitation plays a crucial role in improving functional independence and clinical outcomes within this patient population.

The exercise pressor reflex (EPR) is one mechanism that contributes to increased sympathetic nervous system activity and blood pressure during exercise (Mitchell *et al.*, 1983; Strange *et al.*, 1993; Kaufman & Forster, 1996). This reflex is activated when group III and IV thin fiber muscle afferents are stimulated by mechanical and/or metabolic signals associated with muscle contraction (McCloskey & Mitchell, 1972; Kaufman *et al.*, 1982; Kaufman *et al.*, 1983; Kaufman *et al.*, 1984; Harms *et al.*, 2016). The central projections of the thin fiber muscle afferents synapse in the dorsal horn of the spinal cord onto spinal interneurons which, in turn, project to the medulla of the brainstem where they impact autonomic nervous system activity (Kaufman & Forster, 1996). A large body of evidence suggests that the EPR is exaggerated in patients with HF-rEF (Piepoli *et al.*, 1996; Middlekauff *et al.*, 2000; Middlekauff *et al.*, 2001; Notarius *et al.*, 2001; Middlekauff *et al.*, 2004; Amann *et al.*, 2014; Antunes-Correa *et al.*, 2014; Olson *et al.*, 2014; Witman *et al.*, 2015), and in animal models of HF-rEF (Ansorge *et al.*, 2004; Li *et al.*, 2004; Smith *et al.*, 2005; Koba *et al.*, 2008, 2010; Morales *et al.*, 2012; Schiller *et al.*, 2019), which contributes to sympatho-excitation and impaired oxygen delivery to the contracting muscle (O'Leary *et al.*, 2004; Amann *et al.*, 2014; Ives *et al.*, 2016; Kaur *et al.*, 2018).

The mechanically sensitive portion of the EPR (*i.e.* the mechanoreflex) contributes importantly to the overall EPR exaggeration found in HF-rEF subjects compared to healthy controls (Li *et al.*, 2004; Smith *et al.*, 2005; Koba *et al.*, 2008; Wang *et al.*, 2010; Morales *et al.*, 2012; Antunes-Correa *et al.*, 2014; Witman *et al.*, 2015; Schiller *et al.*, 2019). Further, the mechanoreflex exaggeration appears due, at least in part, to augmented thin fiber muscle afferent, especially group III afferents, responsiveness (Wang *et al.*, 2010) resulting from a sensitization produced by cyclooxygenase (COX) products of arachidonic metabolism (Middlekauff *et al.*, 2008; Morales *et al.*, 2012; Antunes-Correa *et al.*, 2014; Smith *et al.*, 2020). For example, Middlekauff *et al.* (2008) found in patients with HF-rEF that COX inhibition with indomethacin significantly reduced the increase in muscle sympathetic nerve

activity and blood pressure during low-intensity rhythmic hand-grip exercise; a contraction protocol that likely produced a robust mechanical stimulus and only a moderate metabolic stimulus. Morales *et al.* (2012) extended those findings by demonstrating that in rats with HF-rEF, inhibition of the COX-2 isoform but not the COX-1 isoform within hindlimb skeletal muscles reduced the increase in blood pressure and renal sympathetic nerve activity (RSNA) evoked during static hindlimb skeletal muscle stretch. The hindlimb muscle stretch maneuver was originally developed in the cat by Stebbins *et al.* (1988) as a model of mechanoreflex activation isolated from contraction-induced metabolite production. Conversely, they found no effect of COX-1 or COX-2 inhibition in healthy rats. Those findings suggested that COX-2 activity in HF-rEF produced a “chronic” sensitization or priming of mechanically activated channels that occurred even in the absence of skeletal muscle contraction. Further, those findings suggest that pharmacologically targeting the COX-2 pathway would be an effective strategy for HF-rEF patients to reduce the sympatho-excitation associated with the EPR. However, COX-2 inhibition has been linked with an increased risk of atherothrombotic events (Kearney *et al.*, 2006). An alternative therapeutic strategy may be to target the receptors that underlie the COX-mediated mechanoreflex sensitization in HF-rEF.

Prostaglandin E₂ (PGE₂) accounts for the largest proportion of COX-2 products of arachidonic metabolism within skeletal muscle (Berlin *et al.*, 1979). PGE₂ is known to interact with endoperoxide 4 receptors (EP4-R; Southall & Vasko, 2001) and thromboxane A₂ receptors (TxA₂-R; Gluais *et al.*, 2005), both of which are found on sensory neurons (Southall & Vasko, 2001; Kopp *et al.*, 2004; Yamauchi *et al.*, 2013; Butenas *et al.*, 2019), and have been demonstrated to play a role in the exaggerated exercise pressor reflex found in a rat model of simulated peripheral artery disease (Leal *et al.*, 2011; Yamauchi *et al.*, 2013). However, the extent to which the receptors contribute to the COX-mediated chronic mechanoreflex sensitization in HF-rEF is unknown. Accordingly, the primary purpose of this investigation was to determine whether EP4-R and TxA₂-R on the sensory endings of thin fiber muscle afferents contribute to the exaggerated reflex sympathetic nervous system and cardiovascular responses to isolated mechanoreflex activation in a myocardial infarction-induced rat model of HF-rEF. Specifically, we tested the hypothesis that hindlimb arterial injection of either the EP4-R antagonist L-161,982 or the TxA₂-R antagonist daltroban would reduce the exaggerated increase in mean arterial pressure (MAP), RSNA, and heart rate (HR) during 30 s of static hindlimb skeletal muscle stretch in rats with HF-rEF, but not healthy (SHAM operated) rats. Furthermore, greater expression of EP4-Rs and TxA₂-Rs within L₄ and L₅ dorsal root ganglia (DRG) have been implicated in the COX-mediated exaggeration of the exercise pressor reflex in rats with simulated peripheral artery disease (Yamauchi *et al.*, 2013; Butenas *et al.*, 2019). Thus, we also sought to determine whether rats with HF-rEF would have greater EP4-R and TxA₂-R mRNA and protein expression in L₄ and L₅ DRG compared to SHAM rats. Finally, we sought to confirm the findings that COX-2, but not COX-1, expression is elevated within skeletal muscle homogenates from HF-rEF subjects compared to controls (Morales *et al.*, 2012; Smith *et al.*, 2020) and also extend those findings across muscles of different fiber-type composition.

2. Methods

2.1 Ethical Approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University (Protocol #4076) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011). Experiments were performed on ~14–19 week old male Sprague-Dawley rats ($n=54$; Charles River Laboratories). Rats were housed two per cage in temperature (maintained at ~22°C) and light (12–12 hour light-dark cycle running from 7 AM to 7 PM)-controlled accredited facilities with standard rat chow and water provided ad libitum.

2.2 Surgical Procedure

Myocardial infarction (MI) was induced in 32 of the 54 rats by surgically ligating the left main coronary artery (Musch & Terrell, 1992). Briefly, rats were anesthetized initially with a 5% isoflurane-O₂ mixture (Butler Animal Health Supply, Elk Grove Village, IL, and Linweld, Dallas, TX) and maintained subsequently on 2.5% isoflurane-O₂ and then intubated and mechanically ventilated with a rodent respirator (Harvard model 680, Harvard Instruments, Holliston, MA) for the duration of the surgical procedure. A left thoracotomy was performed to expose the heart through the fifth intercostal space, and the left main coronary artery was ligated 1–2 mm distal to the edge of the left atrium with a 6–0 braided polyester suture. The thorax was then closed with 2–0 gut, and the skin was closed with 2–0 silk. Prior to termination of anesthesia, bupivacaine (1.5 mg/kg sc) and buprenorphine (~0.03 mg/kg im) were administered to reduce pain associated with the surgery, along with ampicillin (50 mg/kg im) to reduce the risk of infection. After rats were removed from mechanical ventilation and anesthesia, they were monitored closely for ~6 h post surgery. In the remaining 22 of 54 rats, a sham ligation of the coronary artery was performed in which 6–0 braided polyester suture was passed under the left main coronary artery, but not tied. These rats are referred to as “SHAM” rats from this point forward. Following completion of either MI or SHAM procedures, rats were housed one per cage for ten days to minimize risk of infection of the surgical site. During these ten days, the antibiotic baytril (100 mg/mL) was administered in the drinking water. Following completion of the baytril treatment, rats were housed two per cage as described above. All animals were monitored daily for 14 days following MI or SHAM procedure for changes in behavior, gait/posture, breathing, appetite and body weight.

2.3 Echocardiograph Measurements

Transthoracic echocardiograph measurements were performed with a commercially available system (Logiq S8; GE Health Care, Milwaukee, WI) ~24–96 hours before the final experimental protocol. Briefly, the rats were anaesthetized as described above. Once the rat was fully anesthetized, the isoflurane mixture was reduced to 2% isoflurane-O₂. Following 5 minutes at 2% isoflurane, echocardiograph measurements began. The transducer was positioned on the left anterior chest, and left ventricular dimensions were measured. The left ventricular fractional shortening (FS), ejection fraction (EF), end diastolic (LVEDV), end systolic volume (LVESV), and stroke volume (SV) were determined by echocardiographic measurements as previously described (Craig *et al.*, 2019a).

2.4 Final Surgical Procedures and Experimental Protocol

In vivo experiments were performed between four and six weeks following the MI or SHAM procedure and were successfully completed on 36 rats, (13 SHAM and 23 HF-rEF). Eighteen (9 SHAM, 9 HF-rEF) *in-vivo* experiments were attempted but were unsuccessful for various reasons (*e.g.* hindlimb muscle did not stain blue, preparation was physiologically unstable, etc.). On the day of the experiment, rats were anesthetized as described above. Adequate depth of anesthesia was confirmed by the absence of toe-pinch and blink reflexes. The trachea was cannulated, and the lungs were mechanically ventilated (Harvard Apparatus, Holliston, MA) with a 2% isoflurane-balance O₂ gaseous mixture until the decerebration was completed (see below). The right jugular vein and both carotid arteries were cannulated with PE-50 catheters which were used for the injection of fluids, measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), and sampling of arterial blood gasses (Radiometer). HR was measured by electrocardiogram (AD Instruments). The left superficial epigastric artery was cannulated with a PE-8 catheter whose tip was placed near the junction of the superficial epigastric and femoral arteries and a reversible snare (2–0 silk suture) was placed around the left iliac artery and vein as previously described (Rollins *et al.*, 2019). The left calcaneal bone was severed and linked by string to a force transducer (Grass FT03), which, in turn, was attached to a rack and pinion. A retroperitoneal approach was used to expose bundles of the left renal sympathetic nerve, which were then glued (Kwik-Sil, World Precision Instruments) onto a pair of thin stainless-steel recording electrodes connected to a high impedance probe (Grass Model HZP) and amplifier (Grass P511). Multiunit signals from the renal sympathetic nerve fibers were filtered at high and low frequencies (1 KHz and 100 Hz, respectively) for the measurement of RSNA. Successful RSNA recordings were made in 10 of 13 SHAM rats and 16 of 23 HF-rEF rats.

Upon completion of the initial surgical procedures, rats were placed in a Kopf stereotaxic frame. After administering dexamethasone (0.2 mg i.v.) to minimize swelling of the brainstem, a pre-collicular decerebration was performed in which all brain tissue rostral to the superior colliculi was removed (Smith *et al.*, 2001). Following decerebration, anesthesia was terminated, and the rats' lungs were ventilated with room air. Rats were allowed a minimum of 60 minutes to recover from anesthesia before initiation of any experimental protocol. Experiments were performed on decerebrate, unanesthetized rats because anesthesia has been shown to markedly blunt the mechanoreflex and exercise pressor reflex in the rat (Smith *et al.*, 2001). Body core temperature was measured via a rectal probe and maintained at ~37–38°C by an automated heating system (Harvard Apparatus) and heat lamp. Arterial pH and blood gases were analyzed periodically throughout the experiment from arterial blood samples (~75 µL) and maintained within physiological ranges (pH: 7.35–7.45, PCO₂: ~38–40 mmHg, PO₂: ~100 mmHg) by administration of sodium bicarbonate and/or adjusting ventilation as necessary.

We compared the pressor, cardioaccelerator, and RSNA responses during 30 seconds of static stretch of the triceps surae muscle before and after the injection of either the EP4-R antagonist L-161,982 or the TxA₂-R antagonist daltroban into the arterial supply of the left hindlimb. In detail, at least 60 minutes following the decerebration procedure and

termination of anesthesia, baseline muscle tension was set at ~80–100 g by manually turning the rack and pinion. Baseline blood pressure and heart rate were measured for ~30 seconds. An experienced investigator then initiated the stretch protocol by rapidly turning the rack and pinion to a position which was held constant for 30 s. The investigator aimed to produce a peak tension generation of ~0.7–0.8 kg during each static stretch. Importantly, this level of tension approximates the tension typically developed during static rat hindlimb muscle contraction in the decerebrate rat preparation (Smith *et al.*, 2001). Approximately five minutes following the completion of the first (*i.e.* control) static stretch protocol, and after ensuring that blood pressure had returned to its pre-stretch baseline value, we tightened the iliac snare and injected either the EP4-R antagonist L-161,982 (Tocris Bioscience; 1 µg dissolved in 0.2 ml of 0.15% DMSO saline solution; Yamauchi *et al.*, 2013) or the TxA₂-R antagonist Daltroban (Santa Cruz Biotechnology, Inc.; 80 µg dissolved in 0.4 mL of 1% DMSO saline solution; Leal *et al.*, 2011) into the arterial supply of the hindlimb through the superficial epigastric artery catheter. The receptor antagonists remained snared in the hindlimb circulation for 5 minutes, at which time the iliac snare was released. In EP4-R blockade experiments, the hindlimb was reperfused for 25 min. This dose of L-161,982 and timing of the injection protocol have been shown to reduce the pressor response to static hindlimb muscle contraction in rats with simulated peripheral artery disease (Yamauchi *et al.*, 2013; Rollins *et al.*, 2020). In TxA₂-R blockade experiments, the hindlimb was reperfused for 10 minutes. This dose of daltroban and the timing of the injection protocol have been shown to reduce the pressor response to hindlimb muscle stretch and contraction in rats with simulated peripheral artery disease (Leal *et al.*, 2011; Rollins *et al.*, 2020). Following the 25 or 10 min hindlimb reperfusion period, the static stretch protocol was then repeated exactly as described above. We have recently used these exact protocols to investigate the role played by EP4-R and TxA₂-R in the chronic sensitization of the mechanoreflex present in a rat model of simulated peripheral artery disease (Rollins *et al.*, 2020). Further, we have previously demonstrated that up to 1% DMSO (Rollins *et al.*, 2020) and up to 10 min of hindlimb circulation arrest with an iliac snare followed by a reperfusion period had no effect on the pressor response to rat hindlimb muscle stretch (Sanderson *et al.*, 2019). The tension generated during the post drug maneuvers was matched as closely as possible to that produced during the control stretch. Evans blue dye was then injected into the arterial supply of the hindlimb in the same manner as the drug injections to ensure that the receptor antagonists had access to the triceps surae muscle circulation in all experiments. The triceps surae muscles were observed to stain blue in all experiments for which in vivo data are reported. At the end of all experiments in which RSNA was measured, postganglionic sympathetic nerve activity was abolished with administration of hexamethonium bromide (20 mg/kg iv) to allow for the quantification of background noise as described previously (Kempf *et al.*, 2018). The decerebrate rats were then euthanized with an injection of saturated (>3 mg/kg) potassium chloride. The lungs were then excised and weighed. The heart was excised and the atria and right ventricle (RV) were separated from the left ventricle (LV) and septum, and the RV and LV were weighed. In rats with HF-rEF, the LV infarction surface area was measured using planimetry and expressed as %LV endocardial surface area as described previously (Craig *et al.*, 2019b).

2.5 Tissue Collection and Protein/mRNA Isolation

Upon completion of *in-vivo* experiments, the left and right L₄ and L₅ DRG and muscle tissue samples (~20 mg) from the soleus and the white portion of the gastrocnemius muscle of the right hindlimb (*i.e.* the hindlimb not studied *in-vivo*) were harvested as described previously (Butenas *et al.*, 2019). DRG and muscle tissues were collected in both successful and unsuccessful *in-vivo* experiments resulting in a tissue bank from a total of 54 rats. Samples were isolated into 2 mL bead mill tubes containing ~0.5g of 1.4 mm ceramic beads, 350 μ L of RP1 lysis buffer (Macherey-Nagel), and 3.5 μ L of β -Mercaptoethanol, and homogenized for 1 min at 5 m/s using Bead Mill 4 (Fisherbrand™). Total protein and mRNA from tissues were prepared with the Nucleospin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Total Protein and RNA concentrations were determined using the Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA).

2.6 Western blot experiments

Protein samples (30 μ g) were separated on 4–12% Bis-Tris Protein Gels (Invitrogen™) by gel electrophoresis in MES running buffer (Invitrogen™) employing 200 V for 22 min. Gels were then transferred to mini-PVDF membranes using the iBlot 2 Dry Transfer Device (Invitrogen™). Membranes were incubated for ~3 hours with the iBind device with iBind solution (Invitrogen™) with either primary antibodies anti-COX-1 diluted 1:500 (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no. SC-19998; RRID: AB_627283), anti-COX-2 diluted 1:500 (Cayman Chemical Co., Ann Arbor, MI, USA; cat. no. 160112; RRID: AB_10078980), anti-EP4 diluted 1:250 (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no. SC-55596; RRID: AB_2174775), or anti TXA2R diluted 1:250 (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no. SC-515033; RRID: AB_2847878), along with a Goat anti-Mouse IgG (H+L), secondary antibody conjugated with Horse Radish Peroxidase diluted 1:1,000 (ThermoFisher Scientific; Rockford, IL, USA; cat. no. 31430; RRID: AB_228307). Membranes were then incubated for 5 min with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher) and imaged with C-DiGit® Blot Scanner (Li-Cor). Next the membranes were incubated with anti-GAPDH diluted 1:1,000 (ThermoFisher Scientific; Rockford, IL, USA; cat. no. MA5-15738; RRID: AB_10977387) and with Goat anti-Mouse IgG (H+L), secondary antibody conjugated with Horse Radish Peroxidase diluted 1:1,000 (ThermoFisher Scientific; Rockford, IL, USA; cat. no. 31430; RRID: AB_228307) and imaged using the same protocol described above to ensure equal protein loading. The protein bands were quantified and analyzed using the Image Studio software (Li-Cor). In some SHAM rats and some rats with HF-rEF, in western blot experiments for COX-1 and COX-2, detection of protein bands was not discriminable from background. These rats were not included in the data analysis.

2.7 Quantitative Reverse Transcriptase Polymerase Chain Reaction Experiments

Complementary DNA (cDNA) was synthesized from RNA isolates (see above) using the High Capacity RNA-cDNA™ kit (ThermoFisher) according to the manufacturer's instructions as described previously (Copp *et al.*, 2016a). Quantitative reverse transcriptase polymerase chain Reaction experiments were then performed on the cDNA samples using

TaqMan gene expression assays specific for: COX-1 with forward primer: 5'-AGACAGCCGCATCTTCTTGT-3' and reverse primer 5'-CCACAGTCTTCTGAGTGGCA-3', COX-2 (Sequence proprietary; Assay ID: Rn01483828_m1), EP4-R (Sequence proprietary; Assay ID: Rn00583420_m1), and TxA₂-R (Sequence proprietary; Assay ID: Rn00690601_m1), and GAPDH with forward primer: 5'-ACCGCCTGTTGCGTGTTA-3' and reverse primer: 5'-CAATCGCCAACGCCTCAA-3'. All samples were run in triplicate for the gene of interest, and the endogenous control (GAPDH). The results were analyzed with the comparative threshold (C_t) method.

2.8 Data analysis

Muscle tension, blood pressure, HR and RSNA were measured and recorded in real time with a PowerLab and LabChart data acquisition system (AD Instruments). Baseline mean arterial pressure (MAP), RSNA and HR were determined from the 30-second baseline periods that preceded each maneuver. The peak increase in MAP (peak MAP), RSNA (peak RSNA), and HR (peak HR) during static stretch were calculated as the difference between the peak values wherever they occurred during the maneuvers and their corresponding baseline value. The tension-time indexes (TTIs), integrated RSNA ($\int$ RSNA) for the entire 30 s stretch maneuver, $\int$ RSNA for the first 10 s of the stretch maneuver ($\int$ RSNA_{10sec}) and blood pressure indexes (BPIs) were calculated by integration of the area under curve during the stretch maneuver and subtracting the integrated area under the curve during the baseline period. We calculated the peak RSNA and the $\int$ RSNA_{10sec} because the renal sympathetic nerve response to static stretch is often characterized by large bursts early (*i.e.* the first ~10 s) in the maneuver followed by a return in RSNA to near baseline levels for the remainder of the maneuver. All data were assessed for normality, equal variance and/or effectiveness of pairing as appropriate. Baseline MAP, baseline HR, Peak MAP, Peak HR, Peak RSNA, BPI, $\int$ RSNA, $\int$ RSNA_{10sec}, and TTIs were compared with Sidak multiple comparison tests. Data for echocardiograph measurements, body and organ weights, heart morphometrics, Western blots, and polymerase chain reaction experiments were analyzed with unpaired Student's *t*-tests or Mann-Whitney U tests as appropriate. Two outliers were detected (Grubbs' Test for Outliers) in the HF-rEF group for COX-2 mRNA (51.7 and 15.4 a.u.; $P < 0.05$) in the white portion of the gastrocnemius muscle. These values were removed from the analysis and data shown in Fig. 6D. Importantly, removal of these values did not alter the interpretation of our primary finding compared to when these values were included. Data are expressed as mean $\pm$ SD. Statistical significance was defined as $P \leq 0.05$.

3. Results

3.1 Body weights and heart morphometrics

Body weights were not different between SHAM and HF-rEF rats (Table 1). The lung weight to body weight ratio along with LVEDV and LVESV were significantly larger, whereas fractional shortening and ejection fraction were significantly lower in HF-rEF compared to SHAM rats. Lastly, RV to body weight and LV to body weight ratios along with stroke volume were not different between SHAM rats and rats with HF-rEF.

3.2 Effect of HF-rEF on the mechanoreflex

Pooled data from control conditions across experimental groups in the present investigation (*i.e.* 13 SHAM and 23 HF-rEF rats) indicate that, compared to SHAM rats, HF-rEF rats had larger peak MAP (SHAM: 14 ± 8 ; HF-rEF: 26 ± 12 mmHg; $P < 0.01$), BPI (SHAM: 209 ± 142 ; HF-rEF: 549 ± 403 mmHg·s; $P < 0.01$), and peak HR (SHAM: 9 ± 7 ; HF-rEF: 14 ± 10 bpm; $P < 0.05$) responses to 30 seconds of static stretch. In pooled data from rats in which RSNA was successfully recorded (*i.e.* 10 SHAM and 16 HF-rEF rats) we found no difference in the $\int$ RSNA for the entire 30 s stretch maneuver between groups (SHAM: 245 ± 104 ; HF-rEF: 363 ± 246 a.u.; $P = 0.17$). However, both the peak increase in RSNA (peak RSNA SHAM: $42 \pm 32\%$; HF-rEF: $108 \pm 80\%$; $P < 0.01$) and the $\int$ RSNA for the first 10 s (*i.e.* $\int$ RSNA_{10sec}, SHAM: 105 ± 91 , HF-rEF: 241 ± 231 a.u.; $P = 0.045$) of the stretch maneuver were significantly larger in HF-rEF compared to SHAM rats. The TTI of the static stretch maneuvers were not different between groups (SHAM: 15 ± 4 , HF-rEF: 17 ± 3 kg·s; $P = 0.20$).

3.3 Effect of EP4-R blockade on the mechanoreflex

In SHAM ($n=6$) and HF-rEF ($n=9$) rats, we found that injection of the EP4-R antagonist L-161,982 into the arterial supply of the hindlimb had no effect on peak MAP, BPI, peak HR, peak RSNA, or $\int$ RSNA_{10sec} response to static stretch (Fig. 1). The TTIs of the stretch maneuvers were not different between control and EP4-R blockade conditions in SHAM or HF-rEF rats. Baseline MAP was significantly reduced in HF-rEF rats ($P = 0.03$), but not SHAM rats ($P = 0.057$) after EP4-R blockade, whereas baseline HR was not different between conditions in either SHAM or HF-rEF rats (Table 2; $P > 0.59$ for both).

3.4 Effect of TxA₂-R blockade on the mechanoreflex

In SHAM ($n=7$) and HF-rEF ($n=14$) rats, we found that injection of the TxA₂-R antagonist daltroban into the arterial supply of the hindlimb had no effect on peak MAP, BPI, peak HR, peak RSNA, or $\int$ RSNA_{10sec} response to static stretch (Fig. 2). The TTIs of the stretch maneuvers were not different between control and TxA₂-R blockade conditions in either SHAM rats or HF-rEF rats. Baseline MAP and baseline HR were not different between conditions in SHAM or HF-rEF rats (Table 2; $P > 0.60$ for all). Figure 3 shows an original tracing of the RSNA, pressor and cardioaccelerator response to static stretch before and after the injection of the TxA₂-R antagonist daltroban into the arterial supply of the hindlimb in a rat with HF-rEF.

3.5 DRG EP4-R and TxA₂-R protein and mRNA expression

There was no difference in TxA₂-R protein or mRNA expression within L₄ and L₅ DRG tissue between SHAM and HF rats (Fig. 4A&B). Similarly, there was no difference in EP4-R protein or mRNA expression within L₄ and L₅ DRG tissue between groups (Fig. 4C&D).

3.6 Skeletal muscle COX protein and mRNA expression

There was no difference in COX-1 protein or mRNA expression in either the soleus or white portion of the gastrocnemius muscle between SHAM and HF-rEF rats (Fig. 5). Furthermore, there was no difference in COX-2 protein or mRNA expression in the soleus muscle

between SHAM and HF-rEF rats (Fig. 6A&B). However, COX-2 protein expression was significantly greater within the white portion of the gastrocnemius muscle in HF-rEF rats compared to SHAM rats (Fig. 6C). Lastly, COX-2 mRNA expression was not different within the white portion of the gastrocnemius muscle between SHAM and HF-rEF rats (Fig. 6D).

4. Discussion

We investigated the roles played by EP4-R and TxA₂-R, receptors for COX products of arachidonic acid metabolism, in the chronic sensitization of the mechanoreflex in HF-rEF rats. We found that the injection of an EP4-R antagonist or a TxA₂-R antagonist into the arterial supply of the hindlimb had no effect on the pressor, cardioaccelerator, or RSNA response to static hindlimb muscle stretch in SHAM or HF-rEF rats. Additionally, we found no difference in EP4-R or TxA₂-R protein or mRNA expression in L₄ and L₅ DRG between SHAM and HF-rEF rats. The present findings do not support a role for either EP4-R or TxA₂-R in the chronic mechanoreflex sensitization present in rats with HF-rEF.

The experimental protocols used in this investigation (*i.e.* the receptor antagonists, the dose of the antagonists, and the timing of the post injection maneuvers) have been used recently by our laboratory (Rollins *et al.*, 2020) and others (Leal *et al.*, 2011; Yamauchi *et al.*, 2013) to investigate the role played by EP4-R and TxA₂-R in the mechanoreflex and exercise pressor reflex in healthy rats and rats with femoral artery ligation-induced simulated peripheral artery disease. Specifically, injection of 1 µg of the EP4-R antagonist L-161,982 into the arterial supply of the hindlimb of rats in rats with simulated peripheral artery disease reduced the pressor response to static hindlimb muscle contraction (Yamauchi *et al.*, 2013; Rollins *et al.*, 2020). Moreover, injection of 80 µg of the TxA₂-R antagonist daltroban into the arterial supply of the hindlimb of rats with ligated femoral arteries reduced the pressor response to static (Leal *et al.*, 2011) and dynamic (Rollins *et al.*, 2020) hindlimb muscle stretch and static hindlimb muscle contraction (Leal *et al.*, 2011). Collectively, these findings demonstrate the efficacy of the protocols used in the present investigation to produce effective blockade of EP4-R and TxA₂-R on the sensory endings of thin fiber muscle afferents in rats.

Our finding in sham rats that the injection of the EP4-R or the TxA₂-R antagonist into the arterial supply of the hindlimb had no effect on the pressor, RSNA, or cardioaccelerator response to static stretch is consistent with multiple investigations which have shown that COX metabolites are not required to produce reflex cardiovascular responses in healthy subjects (Morales *et al.*, 2012; Muller *et al.*, 2015; Drew *et al.*, 2017; Butenas *et al.*, 2019; Rollins *et al.*, 2019). More specifically, the present lack of effect of the EP4-R antagonist in SHAM rats is consistent with the recent findings of Yamauchi *et al.* (2013) using the same mode of mechanoreflex activation, and our laboratory using a dynamic mode of mechanoreflex activation (Rollins *et al.*, 2020) in healthy control rats. Moreover, the present lack of effect the TxA₂-R antagonist in SHAM rats is consistent with our recent finding using dynamic mechanoreflex activation in healthy control rats (Rollins *et al.*, 2020). In contrast, however, Leal *et al.* (2011) found that TxA₂-R blockade reduced the pressor response to static hindlimb muscle stretch in healthy rats. The reason for the difference

between the present investigation and that of Leal *et al.* (2011) is unknown, although a substantial difference in the magnitude of pressor response to static stretch in the control condition is noteworthy (~12 in the present investigation vs. ~30 mmHg in Leal *et al.*, 2011). Nevertheless, the results of the present investigation, in corroboration with the bulk of available data described above, indicate that, in healthy subjects, involvement of the COX pathway, including EP4-R and TxA₂-R stimulation, is not necessary to produce reflex sympathetic and cardiovascular responses to a mechanical stimulus.

Consistent with previous findings from other laboratories (Smith *et al.*, 2005; Koba *et al.*, 2008; Morales *et al.*, 2012; Wang *et al.*, 2012) we found that the pressor, RSNA, and cardioaccelerator responses to static stretch were greater in rats with HF-rEF compared to the responses found in SHAM rats. The findings suggest that the development of HF-rEF results in an exaggeration of the mechanoreflex. Our present findings that injection of the EP4-R antagonist or the TxA₂-R antagonist had no effect on the reflex responses to static stretch in HF-rEF rats do not support a role for those receptors in a chronic sensitization or priming of the mechanically activated channels that underlie mechanoreflex activation. Our findings should not be interpreted to suggest that EP4-Rs or TxA₂-Rs do not play a role in the exaggerated EPR in HF-rEF. Indeed, multiple studies at least indirectly support a role for COX metabolites, and by extension COX metabolite receptors, in the reflex control of blood pressure in heart failure during skeletal muscle contractions (Middlekauff *et al.*, 2008; Antunes-Correa *et al.*, 2014; Smith *et al.*, 2020). The possibility remains that acute increases in metabolite production during skeletal muscle contraction interact with EP4-Rs and/or TxA₂-Rs and which results in an “acute” mechanoreflex sensitization. Such an acute sensitization may work additively with chronic mechanoreflex sensitization to produce the overall mechanoreflex exaggeration present during skeletal muscle contractions. Moreover, stimulation of EP4-Rs and/or TxA₂-Rs during skeletal muscle contraction may contribute to the activation of the metabolically sensitive component of the EPR (*i.e.* the metaboreflex) in HF-rEF rats. These possibilities warrant further investigation.

How may our present finding of a lack of effect of the EP4-R and TxA₂-R antagonists on the pressor response to static stretch in HF-rEF rats fit with the recent finding of Morales *et al.* (2012) that COX-2 inhibition reduced the pressor and RSNA response to static stretch in HF-rEF rats? There are at least a couple of possible explanations. First, redundancy among various receptors may have masked any possible contribution of either EP4-Rs or TxA₂-Rs when the receptors were antagonized independently in our experiments (Stone *et al.*, 2015a). Second, COX-2-mediated, chronic mechanoreflex sensitization in HF-rEF may occur through endoperoxide-3 subtype C (EP3C) receptors. For example, of the EP receptors identified in rat DRG (*i.e.* EP1, EP2, EP3C, and EP4), only stimulation of EP3C and EP4, were found to evoke PGE₂-induced sensitization of DRG cells *in-vitro* (Southall & Vasko, 2001). In the present investigation, we chose to investigate EP4-Rs and TxA₂-Rs, and not EP3C-Rs, because chronic exercise training was found recently to reduce the mechanoreflex in HF-rEF patients which was associated with concurrent reductions in COX-2, EP4-R, and TxA₂-R, but not EP1–3 receptor, mRNA from skeletal muscle homogenates (Antunes-Correa *et al.*, 2014). Nevertheless, the possibility remains that EP3C receptors may contribute to the COX-2 mediated, chronic mechanoreflex sensitization in HF-rEF.

To our knowledge, the present investigation is the first to report the effect of heart failure on EP4-R and TxA₂-R mRNA and protein expression in DRG neurons. Our finding that EP4-R and TxA₂-R mRNA and protein expression were not different between SHAM and HF-rEF rats was surprising given the fact that exercise training reduced EP4-R and TxA₂-R mRNA expression in skeletal muscle homogenates from HF-rEF patients (Antunes-Correa *et al.*, 2014). This discrepancy may highlight the value of investigating sensory receptor expression in DRG rather than, or at least in addition to, skeletal muscle homogenates. Although the fact that Antunes-Correa *et al.* (2014) compared values between exercise trained and untrained HF-rEF patients whereas we compared values between healthy control and HF-rEF rats must also be considered. Interestingly, we have reported recently that, compared to control rats, rats with simulated peripheral artery disease develop COX-mediated chronic mechanoreflex sensitization concurrent with increased EP4-R and TxA₂-R protein expression in lumbar DRG (Butenas *et al.*, 2019). Considered in conjunction with those recent findings (Butenas *et al.*, 2019), the present results indicate that different rat models of cardiovascular disease (*i.e.* heart failure versus simulated PAD) demonstrate different effects on EP4-R and TxA₂-R expression in DRG and different mechanisms of COX-mediated chronic mechanoreflex sensitization.

Our finding that COX-1 protein and mRNA expression in the soleus muscle (high oxidative capacity, ~91% type I and IIA muscle fibers; Delp & Duan, 1996) and the white portion of the gastrocnemius muscle (low oxidative capacity, 100% type IIB and D/X muscle fibers; Delp & Duan, 1996) were not different between SHAM and HF-rEF rats is consistent with, and extend, recent findings from vastus lateralis muscle samples from HF-rEF patients (Antunes-Correa *et al.*, 2014) and only the white portion of the gastrocnemius muscle of rats with HF-rEF (Morales *et al.*, 2012). Moreover, consistent with previous findings (Morales *et al.*, 2012), we found that COX-2 protein expression in the white portion of the gastrocnemius muscle was greater in HF-rEF rats compared to SHAM rats. We found no difference in COX-2 protein expression in the soleus muscle, however, which suggests important fiber-type selectivity regarding the effect of HF-rEF on COX-2 protein expression, at least in the rat. It is also noteworthy that COX-2 protein expression was found recently to be elevated in skeletal muscle of HF-rEF patients compared to control counterparts (Smith *et al.*, 2020). We were surprised to find that COX-2 mRNA expression in the white portion of the gastrocnemius muscle was not different between SHAM and HF-rEF rats especially when considering the recent findings of Antunes-Correa *et al.* (2014) who reported that exercise training decreased COX-2 mRNA (and protein expression) in vastus lateralis muscle samples from HF-rEF patients, although a healthy-control group was not included in that investigation. Our findings appear to suggest that the elevated COX-2 protein expression in the white portion of the gastrocnemius muscle in HF-rEF rats results primarily from post-transcriptional modification. That interpretation warrants caution, however, given that we found a large amount of variability in the COX-2 mRNA measurement which included two HF-rEF samples that were statistical outliers (see Methods). Nevertheless, the finding that, on average, COX-2 mRNA in the white portion of the gastrocnemius muscle was not different between SHAM and HF-rEF rats is interesting and requires further investigation in healthy control and heart failure patients.

Several experimental considerations should be noted. First, we attempted experiments to challenge the efficacy of EP4-R and TxA₂-R blockade in which we hypothesized that L-161,982 and daltroban would reduce a pressor response produced by the injection of an appropriate receptor agonist into the arterial supply of the hindlimb. However, we did not observe pressor responses, and often observed marked depressor responses, following the injection of either PGE₂ (an EP4-R agonist) or U-46619 (a TxA₂-R agonist; Leal *et al.*, 2011) into the arterial supply of the rat hindlimb (unpublished observations). Thus, as indicated above, we must rely on previous findings from our laboratory (Rollins *et al.*, 2020) and others (Leal *et al.*, 2011; Yamauchi *et al.*, 2013) using these same protocols as evidence that EP4-R and TxA₂-R were likely blocked by their respective antagonists in our experiments. Second, we stimulated the mechanically-activated channels on thin fiber muscle afferents by passively stretching rat hindlimb skeletal muscles whereas during exercise those channels are stimulated when skeletal muscles contract and shorten and intramuscular pressure increases (Gallagher *et al.*, 2001). Skeletal muscle stretch, however, appears to be a useful model of isolated mechanoreflex activation. Specifically, Kaufman *et al.* (1983) demonstrated that hindlimb muscle stretch did not activate afferents that responded to noxious stimuli, at least in the cat, and the vast majority (*i.e.* >86%) of the muscle afferents that respond to rat hindlimb muscle stretch also respond to hindlimb muscle contraction (Stone *et al.*, 2015b). Moreover, studies that successfully identify a mechanism that contributes to the generation of the pressor response evoked during rat hindlimb muscle stretch have also found that same mechanism contributes to the generation of the pressor response evoked during hindlimb muscle contraction (Smith *et al.*, 2003; Smith *et al.*, 2005; Nakamoto & Matsukawa, 2008; Leal *et al.*, 2011; McCord *et al.*, 2011; Tsuchimochi *et al.*, 2011; Yamauchi *et al.*, 2012; Wang *et al.*, 2013; Copp *et al.*, 2016b, a; Downey *et al.*, 2017; Kim & Kaufman, 2019; Schiller *et al.*, 2019). Third, baseline MAP was significantly reduced following EP4-R blockade in HF-rEF rats and there was a trend ($P = 0.057$) towards a reduction in SHAM rats. The effect on baseline MAP was surprising given the recent findings that the same EP4-R blockade protocol had no effect on baseline MAP in control rats or rats with a chronically ligated femoral artery (Yamauchi *et al.*, 2013; Rollins *et al.*, 2020). It is unlikely that the lower baseline MAP impacted the present findings given that the baseline MAP following injection of the EP4-R antagonist in both SHAM and HF-rEF rats did not suggest poor physiological status (*i.e.* they were not abnormally low). Moreover, there was no correlation between baseline MAP and the peak delta MAP response to stretch in either SHAM ($r = -0.22$; $P = 0.73$) or HF-rEF ($r = -0.09$; $P = 0.22$) rats in the present investigation which suggests that differences in baroreflex modulation of the pressor response did not exist across the relatively narrow baseline MAP range that we observed. Finally, given our interrogation of EP4-R and TxA₂-R protein expression in whole DRG tissue, we cannot rule out a contribution of afferents other than muscle afferents (e.g., cutaneous) to the protein signal.

5. Conclusions

In summary, we investigated the role played by EP4-Rs and TxA₂-Rs, receptors associated with products of COX activity, in the chronic mechanoreflex sensitization present in HF-rEF. We found that injection of an EP4-R antagonist or a TxA₂-R antagonist into the arterial

supply of the hindlimb had no effect on the pressor, cardioaccelerator, or RSNA response to static hindlimb skeletal muscle stretch in sham or rats with HF-rEF. Our data do not support a role for either EP4-Rs or TxA₂-Rs in the chronic mechanoreflex sensitization present in the in the rat myocardial infarction model of HF-rEF.

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New Findings:

- What is the central question of this study?

Do endoperoxide 4 and thromboxane A₂ receptors, receptors for cyclooxygenase products of arachidonic metabolism, on thin fiber muscle afferents play a role in the chronic mechanoreflex sensitization present in rats with heart failure with reduced ejection fraction?
- What is the main finding and its importance?

Our data do not support a role for endoperoxide 4 receptors or thromboxane A₂ receptors in the chronic mechanoreflex sensitization in HF-rEF rats.

Figure 1:

The peak mean arterial pressure (peak MAP; A), peak renal sympathetic nerve activity (peak RSNA; B), peak heart rate (peak HR; C), blood pressure index (BPI, D), and the first 10 seconds of the integrated change in RSNA ($\int$ RSNA_{10sec}, E) in response to 30 seconds of static hindlimb muscle stretch before (Control) and after injection of the EP4-R antagonist L-161,982 (1 µg) into the arterial supply of the hindlimb of SHAM ($n=6$) and HF-rEF ($n=9$) rats. The tension-time index (TTI) was not different between conditions for either group (F). Data were analyzed with Sidak multiple comparisons tests and are expressed as mean $\pm$ SD overlaid with individual responses.

Figure 2:

The peak mean arterial pressure (peak MAP; A), peak renal sympathetic nerve activity (peak RSNA; B), peak heart rate (peak HR; C), blood pressure index (BPI, D), and the first 10 seconds of the integrated change in RSNA ($\int \text{RSNA}_{10\text{sec}}$, E) responses to 30 seconds of static hindlimb muscle stretch before (Control) and after injection of the TxA_2 -R antagonist Daltroban (80 μg) into the arterial supply of the hindlimb of SHAM ($n=7$) and HF-rEF ($n=14$) rats. The tension-time index (TTI) was not different between conditions for either group (F). Data were analyzed with Sidak multiple comparisons tests and are expressed as mean $\pm$ SD overlaid with individual responses.

Figure 3:

Example of original tracings of the renal sympathetic nerve activity (RSNA), blood pressure (BP) and heart rate (HR) responses to 30 seconds of static hindlimb muscle stretch in a rat with HF-rEF (ejection fraction: 58%) before and after administration of the TxA₂-R antagonist daltroban into the arterial supply of the hindlimb (*i.e.* Control and Post Daltroban).

Figure 4:

Endoperoxide 4 receptor (EP4-R) protein (A, SHAM $n=12$, HF-rEF $n=15$) and mRNA (B, SHAM $n=13$, HF-rEF $n=15$) expression in L₄ and L₅ dorsal root ganglia (DRG) were not different between groups. Likewise, thromboxane A₂ receptor (TxA₂-R) protein (C, SHAM $n=12$, HF-rEF $n=15$) and mRNA (D, SHAM $n=12$, HF-rEF $n=16$) expression in L₄ and L₅ DRG were not different between groups. GAPDH was used as loading controls and a reference gene sample. Examples of original western blot images from two sham and two HF-rEF rats are shown above respective graphs. Data are mean $\pm$ SD with individual values.

Figure 5:

Cyclooxygenase (COX)-1 protein expression within the soleus muscle (A, SHAM $n=8$, HF-rEF $n=8$) and white portion of the gastrocnemius muscle (C, SHAM $n=4$, HF-rEF $n=5$) were not different between groups. Likewise, COX-1 mRNA expression within the soleus muscle (B, SHAM $n=7$, HF-rEF $n=7$) and white portion of the gastrocnemius muscle (D, SHAM $n=5$, HF-rEF $n=8$) were not different between groups. GAPDH was used as loading controls and a reference gene sample. Examples of original western blot images from two sham rats and two HF-rEF rats are shown above respective graphs. Data are mean $\pm$ SD with individual values.

Figure 6:

Cyclooxygenase (COX)-2 protein expression within the soleus muscle (A, SHAM $n=11$, HF-rEF $n=16$) was not different between groups, whereas COX-2 protein expression within the white portion of the gastrocnemius muscle was significantly greater (*) in rats with HF-rEF ($n=15$) compared to SHAM ($n=10$, C). COX-2 mRNA expression within the soleus muscle (B, SHAM $n=9$, HF-rEF $n=11$) and the white portion of the gastrocnemius muscle (D, SHAM $n=8$, HF-rEF $n=11$) were not different between groups. GAPDH was used as loading controls and a reference gene sample. Examples of original western blot images for two SHAM rats and two HF-rEF rats are shown above respective graphs. Data are mean $\pm$ SD with individual values.

Table 1.

Weights, and heart morphometrics in SHAM and HF-rEF rats

	SHAM (<i>n</i> =22)	HF-rEF (<i>n</i> =32)	P-Value
Body weight (g)	503 ± 47	494 ± 43	0.454
Lung/body weight (mg/g)	3.39 ± 0.44	3.87 ± 1.17	0.049
RV/body weight (mg/g)	0.54 ± 0.07	0.58 ± 0.19	0.228
LV/body weight (mg/g)	1.88 ± 0.20	1.79 ± 0.50	0.226
LV EDV (mL)	1.24 ± 0.30	2.06 ± 0.59	<0.001
LV ESV (mL)	0.22 ± 0.09	1.21 ± 0.47	<0.001
Stroke volume (mL)	0.98 ± 0.25	0.88 ± 0.22	0.072
Fractional shortening (%)	48 ± 6	20 ± 6	<0.001
Ejection fraction (%)	83 ± 6	45 ± 11	<0.001
Infarct size (%)	-	26 ± 9	-

Values are means ± SD. LV, left ventricle; RV, right ventricle; EDV, end diastolic volume; ESV, end systolic volume. Data were compared using student's t-test.

Table 2.

Baseline MAP and HR in SHAM and HF-rEF rats.

	MAP (mmHg)	HR (bpm)
SHAM (<i>n</i> =6)		
Control	102 ± 10	490 ± 29
Post L-161,982	91 ± 6 [‡]	482 ± 18
HF-rEF (<i>n</i> =9)		
Control	103 ± 11	487 ± 17
Post L-161,982	93 ± 17 [*]	479 ± 31
SHAM (<i>n</i> =7)		
Control	86 ± 18	469 ± 33
Post Daltroban	93 ± 12	476 ± 43
HF-rEF (<i>n</i> =14)		
Control	79 ± 14	483 ± 36
Post Daltroban	89 ± 13	488 ± 33

Values are means ± SD. MAP, mean arterial pressure; HR, heart rate. Data were compared with Sidak multiple comparisons tests.

^{*}P < 0.05 vs Control;

[‡]P = 0.057 vs. Control.