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Research article

Decline of cardiomyocyte contractile performance and bioenergetic function in socially stressed male rats

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ABSTRACT

Chronic social stress has been epidemiologically linked to increased risk for cardiovascular disease, yet the underlying pathophysiological mechanisms are still largely elusive. Mitochondrial (dys)function represents a potential intersection point between social stress exposure and (mal)adaptive cardiac responses. In this study, we used a rodent model of social stress to study the extent to which alterations in the cellular mechanical properties of the heart were associated with changes in indexes of mitochondrial function. Male adult rats were exposed to repeated episodes of social defeat stress or left undisturbed (controls). ECG signals were recorded during and after social defeat stress. Twenty-four hours after the last social defeat, cardiomyocytes were isolated for analyses of mechanical properties and intracellular Ca^{2+} dynamics, mitochondrial respiration, and ATP content. Results indicated that social defeat stress induced potent cardiac sympathetic activation that lasted well beyond stress exposure. Moreover, cardiomyocytes of stressed rats showed poor contractile performance (e.g., slower contraction and relaxation rates) and intracellular Ca^{2+} derangement (e.g., slower Ca^{2+} clearing), which were associated with indexes of reduced reserve respiratory capacity and decreased ATP production. In conclusion, this study suggests that repeated social stress provokes impaired cardiomyocyte contractile performance and signs of altered mitochondrial bioenergetics in the rat heart. Future studies are needed to clarify the causal link between cardiac and mitochondrial functional remodeling under conditions of chronic social stress.

1. Introduction

Chronic social stress is perceived as one of the most intense forms of stress encountered by people in daily life (Almeida, 2005) and has been linked to greater risk for cardiovascular disease (CVD) (e.g., Dimsdale, 2008; Osborne et al., 2020). Yet, studies on chronic psychosocial stress and CVD in humans are generally more epidemiological than pathophysiological in nature. Indeed, social stress is often associated with negative health behaviors (e.g., increased rates of smoking, sedentary lifestyle) that influence cardiovascular function and confound efforts to assess the direct impact of social stress on the heart (Osborne et al., 2020). Rat models based on naturalistic social stress paradigms provide a tool to examine the cardiac consequences of chronic social stress exposure and the underlying pathophysiology in the absence of confounding variables. For example, previous studies have reported that exposure to repeated episodes of social defeat, a highly translational social stress model that induces robust cardiovascular responses (Sgoifo et al., 1999), is associated with electrical and mechanical defects in the whole heart that may predispose to enhanced CVD risk (Carnevali et al., 2013; Andolina et al., 2021). However, a deeper investigation of the impact of social defeat stress at the cardiac cellular and sub-cellular level is needed for elucidating potential pathophysiological mechanisms leading to

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stress-related CVD. Recently, adverse remodeling in the heart of male rats exposed to repeated bouts of social defeat has been associated with non-coding RNA (i.e., overexpression of microRNA-34a) and transcriptomic profile changes that hint at compromised mitochondrial efficiency as a putative underlying pathophysiological process (Andolina et al., 2021).

Mitochondrial (dys)function represents a potential intersection point between social stress exposure and (mal)adaptive cardiac remodeling. Mitochondria influence cardiomyocyte physiology by regulating bioenergetics, redox signaling, oxidative stress, calcium handling, contractile properties, and apoptosis. As key components of the stress response, mitochondria are primarily responsible for meeting the enormous energy demands of the "fight or flight" response (e.g., increased heart rate) in vital tissues such as the heart (Manoli et al., 2007; Schubert et al., 2009; Picard et al., 2018), the most metabolically active organ in the body. However, recent conceptual frameworks suggest that prolonged and/or repeated stress may lead to progressive impairment of mitochondrial function that favors cell death through apoptotic signaling (Manoli et al., 2007; Picard and McEwen, 2018; Picard et al., 2018) and leaves one vulnerable to disease, such as CVD (Chistiakov et al., 2018). Nevertheless, the relation between cardiomyocyte and mitochondrial functional decline has hardly been investigated in preclinical stress research and never in the context of social stress. For example, preliminary rat studies have demonstrated that chronic restraint stress induces cardiomyocyte injury alongside suppression of several proteins involved in lipid metabolism and the Krebs cycle within mitochondria (Liu et al., 2004). Relatedly, restraint stress was also found to induce cardiomyocyte apoptosis via dysfunctional mitochondrial energy metabolism (Wang et al., 2009), suggesting that uncompromised mitochondrial function may be critical to maintain healthy cardiomyocytes during chronic stress exposure.

The current study aimed at providing novel insights into the cellular and subcellular remodeling provoked by repeated exposure to social defeat stress. We tested the hypothesis that repeated social stress-induced cardiac sympatho-adrenomedullary activation would lead to poor contractile performance and intracellular calcium derangement in male rats, and explored whether this decline in cardiomyocyte contractile performance would be associated with signs of reduced mitochondrial function. To do so, we investigated (i) heart rate responses during and in the aftermath of stress exposure as indirect measures of cardiac sympathetic activation, (ii) mechanical and intracellular Ca^{2+} properties as indexes of cardiomyocyte contractile performance and calcium handling, and (iii) mitochondrial respiration and cellular ATP content as proxies of mitochondrial function in male rats.

2. Materials and methods

2.1. Animals

Three-month-old male wild-type Groningen rats were used for the experiments. This rat strain, currently bred at the University of Parma and originally derived from the University of Groningen (The Netherlands), shows potent cardiovascular responses to environmental challenges (Sgoifo et al., 2005). Experimental rats (n = 15) were housed individually 1 week before the beginning of the procedures. Older (12-month-old) male wild-type Groningen rats (n = 8) were housed in another room with an oviduct-ligated female partner and used as residents in the resident-intruder paradigm (see below for details). All rats were housed in climate-controlled rooms, with a 12-h light cycle (lights on at 7 pm) and *ad libitum* food and water. Experimental procedures were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n. 449/2017-PR). All efforts were made to reduce sample size and minimize animal suffering.

2.2. Repeated social defeat stress

Rats were randomly assigned to the repeated social defeat (RSD) or control (CTR) group. The RSD protocol, which was based on a classical "resident–intruder" paradigm (Miczek, 1979), was identical to that adopted in our previous study (Andolina et al., 2021). In brief, each intruder was moved to the cage of a resident rat for 30 min after the removal of the resident's female partner. Resident rats were assessed for their level of aggression before the beginning of the study. Intruders were threatened and physically assaulted by the resident rat. After showing signs of social subordination and defeat (i.e., when the intruder rat assumed a supine posture that was held for at least 5 s) or after 15 min, whichever came first, intruders were confined behind a Plexiglas partition within the resident's cage for the remainder of the 30-min period, allowing for exposure to aggressive threats without physical harm. At the end of the social defeat session, which took place between 10:00 and 11:00 am, intruders were brought back to their home cages. Intruders were submitted to eight episodes on consecutive days and were exposed each day to a different resident. CTR rats remained in their home cages in a different room during the social defeat procedure. CTR manipulation consisted of handling for 60 s by the same experimenter and occurred in correspondence of each social defeat episode.

2.3. Assessment of heart rate and locomotor activity responses to social defeat

Two weeks before the beginning of the repeated social defeat stress procedure, RSD rats (n = 5) were implanted with radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN) for recordings of ECG (sampling frequency 1000 Hz) and locomotor activity (LOC) signals under isoflurane anesthesia (2% in 100% oxygen) (Zoetis, Italy). The transmitter body was placed in the abdominal cavity; one electrode was fixed to the dorsal surface of the xyphoid process and another electrode was placed in the anterior mediastinum close to the right atrium, according to the previously described procedure (Sgoifo et al., 1996). Animals were housed individually post-surgery and were prophylactically injected for 2 days with flunixin (Megluflen, Izo, Italy, 0.2 mL/kg, s. c.). ECG and LOC signals were recorded in correspondence of the first and last social defeat episode. Specifically, recordings occurred during (i) the hour that preceded social defeat (baseline period), with the animals in their home cages; (ii) the 30-min social defeat episode; and (iii) the 5 h following social defeat, with the animals back in their home cages. For each recording period, separate estimates of heart rate (HR, reported in beats per minute, bpm) and LOC (reported in counts per minute, cpm) were generated. For HR analysis, ECG signals were visually inspected to ensure that all R-waves were correctly detected. Those parts of ECG recordings which exhibited recording artifacts were discarded without substitution and excluded from further analysis using ChartPro 5.0 software (ADInstruments, Sydney, Australia). Moreover, the area-under-the-curve (AUC) was calculated as HR (reported in beats) and LOC (reported in counts) changes during and after the first and last social defeat episode with respect to ground.

2.4. Assessment of cardiomyocyte and mitochondrial function

2.4.1. Cardiomyocyte isolation

RSD (n = 5) and CTR (n = 5) rats were submitted to the repeated social defeat stress/control procedure as described above. Twenty-four hours after the last social defeat episode/control procedure, rats were decapitated under isoflurane anesthesia and hearts were rapidly excised through median sternotomy and mounted on a Langendorff isolated heart apparatus. Individual left ventricular myocytes were enzymatically isolated by collagenase perfusion in accordance with the previously described procedure (Bocchi et al., 2019). Briefly, the heart was perfused at 37 °C by means of an aortic cannula with: (1) a calcium-free solution

for 5 min to remove blood (126 mM NaCl, 22 mM dextrose, 5.0 mM $MgCl_2$, 4.4 mM KCl, 20 mM taurine, 5 mM creatine, 5 mM Na pyruvate, 1 mM NaH_2PO_4, and 24 mM HEPES; pH = 7.4, adjusted with NaOH); (2) a low-calcium solution (0.1 mM) plus 1 mg/ml type 2 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.1 mg/ml type XIV protease for about 15 min, and (3) an enzyme-free, low-calcium solution for 5 min. All solutions were gassed with 100% oxygen; all chemicals, where not differently stated, were purchased from Merck KGaA, Darmstadt, Germany. The left ventricle was then chopped and agitated for 10 min. The cardiomyocytes were filtered through a nylon mesh and a fraction was re-suspended in low-calcium solutions for 20 min, gradually brought to 1mM Ca^{2+} in about 80 min. The remaining isolated left ventricle myocytes were suspended in a low-calcium solution (0.1 mM); an aliquot was used to assess mitochondrial respiration, while another one was stored at -80 °C for the subsequent evaluation of ATP content.

2.4.2. Mechanical properties and calcium transients

Mechanical properties and calcium transients were evaluated using the IonOptix fluorescence and contractility systems (IonOptix, Milton, MA, USA), as previously described (Savi et al., 2021). Left ventricle myocytes were placed in a chamber mounted on the stage of an inverted microscope (Nikon-Eclipse TE2000-U, Nikon Instruments, Florence, Italy) and superfused (1 ml/min at 37 °C) with a Tyrode solution containing: 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM glucose, and 1.8 mM $CaCl_2$ (pH = 7.4, adjusted with NaOH) (all chemicals from Merck KGaA, Darmstadt, Germany). Only rod-shaped myocytes with clear edges and average sarcomere length >1.7 μm were selected for the analysis. All the selected myocytes did not show spontaneous contractions. The cells were field stimulated at a frequency of 0.5 Hz and 1 Hz by constant current pulses (2 ms in duration, and twice the diastolic threshold in intensity; MyoPacer Field Stimulator, IonOptix). Sampling rate was fixed at 1 kHz. A total of 136 CTR and 127 RSD cells were analyzed from n = 5 rats per group. We computed: mean diastolic sarcomere length, fraction of shortening (FS; %), maximal rates of shortening (-dl/dt_{max}; μm/s) and re-lengthening (+dl/dt_{max}; μm/s), and time of total cycle (T-cycle; s) measured at 10%, 50%, and 90% of re-lengthening.

In a subgroup of 80 CTR and 86 RSD cells, Ca^{2+} transients were determined by epifluorescence after loading the myocytes with 5 μM fluo 3-AM (Thermo Fisher Scientific, Waltman, MA, USA), previously mixed with PluronicTM F-127 (10% final concentration; Thermo Fisher Scientific) for 20 min. Excitation length was 480 nm, with emission collected at 535 nm using a 40x oil objective lens (NA 1:3). The steady-state contraction of myocytes was achieved before data recording by means of a 10-s conditioning stimulation. The sampling rate was set at 1 kHz. Fluo-3 signals were expressed as normalized fluorescence (f/f0: fold increase). The time course of the fluorescence signal decay was described by a single exponential equation, whose time constant (τ) was used as a measure of the rate of intracellular calcium clearing (Bassani et al., 1994). The time to peak of the calcium transients was also measured.

2.4.3. Mitochondrial respiration and ATP content

Respiratory rates of isolated cardiomyocytes from RSD and CTR rats were assayed polarographically at 30 °C using a Clark-type oxygen electrode (Oxygraph System Hansatech Instruments England). Endogenous (coupled) respiration was determined in intact cells suspended in the isolation buffer, containing 22 mM glucose and 5 mM pyruvate as energy substrates, and was sensitive to potassium cyanide. Uncoupled respiration was determined by adding 15 μM of the Carbonyl cyanide m-chlorophenylhydrazone (CCCP) uncoupler. Cells were counted the Bürker chamber and respiratory rates were expressed as nmol O_2/min/10^5 cells.

ATP intracellular content was determined using the Luminescence ATP Detection Assay System (ATPlite; PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. A frozen pellet of isolated cardiomyocytes was resuspended in 1 mL of Phosphate Buffered Saline, followed by an additional twenty-fold dilution. The diluted cell suspension (100 μL/well) was pipetted in triplicate in a 96-well white plate and lysed with 50 μL of mammalian cell lysis solution for 5 min in an orbital shaker. After the addition of the substrate solution (50 μL/well), 5 min of shaking and 10 min of dark-incubation, luminescence intensity was measured by the TECAN SpectraFluor Plus Microplate Reader (TECAN, Männedorf, Switzerland). The row luminescence data, expressed as relative light units (RLUs), were normalized to the total protein content of each sample, measured with the Bradford's method (Bradford, 1976) using the Bio-Rad protein assay and following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

2.5. Statistical analysis

All statistical analyses were performed using the IBM SPSS statistical package (International Business Machines Corporation, Armonk, NY, USA, version 27). Normal distribution of variables was checked by means of the Kolmogorov–Smirnov test. To test for the effects of stress exposure on cardiac chronotropy, a one-way ANOVA for repeated measures was applied (within subject factors: "stress episode" with two levels (first and last episode), and "time" with seven levels (baseline, social defeat, and 1,2,3,4,5 h after social defeat)), followed by pre-planned paired-t-test comparisons also on AUC values. To test for the effects of stress exposure on cardiomyocyte and mitochondrial function, a series of GLM ANOVA for repeated measurements and unpaired Student's t tests were applied, respectively. Data are reported as means ± standard error of the mean (SEM). The statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of repeated social defeat on cardiac chronotropy

ANOVA yielded a significant effect of "time" for HR values during the first ($F = 10.2$; $p < 0.01$) and last ($F = 86.2$, $p < 0.01$) social defeat procedure. As listed in Table 1, during the physical and sensory interaction with an aggressive rat (i.e., social defeat), mean HR was significantly higher compared with the respective baseline value ($p < 0.05$). This tachycardic response (i) was clearly associated with significantly greater levels of LOC activity, and (ii) persisted for 4 h and 3 h ($p < 0.05$) after the return of the rats in their home cages following the first and last social defeat, respectively, in the absence of significant changes in LOC values compared to baseline (Table 1). Moreover, baseline HR values prior to the last social defeat episode were significantly higher compared to baseline HR values prior to the first exposure ($p < 0.05$) (Table 1). Finally, the overall HR and LOC responses to the social defeat procedure,

Table 1. Heart rate (HR) and locomotor activity (LOC) responses to the first and last social defeat (SD) episode.

	HR first SD	HR last SD	LOC first SD	LOC last SD
Baseline (60 min)	344±3	370±5 *	2.5±0.9	4.5±2.2
SD (30 min)	504±7#	477±9#	21.0±3.3#	17.2±4.5#
0-60 min after	415±8#	412±13#	5.8±1.5	6.7±0.8
60-120 min after	416±12#	405±11#	5.1±1.5	4.8±0.8
120-180 min after	420±10#	411±7#	7.4±1.3	4.8±1.0
180-240 min after	412±1#	411±14	6.6±1.7	5.6±1.5
240-300 min after	375±3	402±12	1.8±0.2	6.4±1.8
AUCg	126137±825	124644±2694	2179±146	2019±233

Notes. Data are presented as means ± SEM and expressed in beats per minute (HR), counts per minute (LOC), beats (AUCg HR), and counts (AUCg LOC). AUCg = area under the curve with respect to ground. # indicates a significant difference ($p < 0.05$) compared with the respective baseline value. * vs. indicates a significant difference ($p < 0.05$) compared with the first SD episode.

as calculated as the AUC with respect to ground, did not differ between the first and last exposure (Table 1).

3.2. Effects of repeated social defeat on cardiomyocyte contractility and Ca^{2+} transients

Representative recordings of sarcomere shortening in the two groups of cells stimulated at 0.5 Hz are reported in Figure 1A. The average diastolic sarcomere length was similar in both groups (1.76 ± 0.003 μm in CTR and 1.77 ± 0.003 μm in RSD cells). However, RSD cells exhibited a significant decrease in the maximal rate of shortening (-dl/dt_{max}, -43%, $p < 0.01$, Figure 1B) and re-lengthening (+dl/dt_{max}, -59%, $p < 0.01$, Figure 1C) and in the fraction of shortening (FS, -33%, $p < 0.01$, Figure 1D). Furthermore, RSD cells were characterized by a significant prolongation of the time of T-cycle at 10%, 50%, and 90% of re-lengthening (+19, +28, and +35% respectively, $p < 0.01$; Figure 1E).

Representative traces of calcium transients in cardiomyocytes from CTR and RSD rats are shown in Figure 2. The time required for cytosolic calcium removal was significantly longer in RSD cells (+28%; $p < 0.015$) (Figure 2B). No significant group differences were observed in the amplitude (f/f0, fold increase) and the time to peak of Ca^{2+} transients (Figure 2C and D).

3.3. Effects of repeated social defeat on mitochondrial respiration and cellular ATP content

Respiratory rates and ATP content in cardiomyocytes of RSD and CTR rats are depicted in Figure 3. No significant group differences were

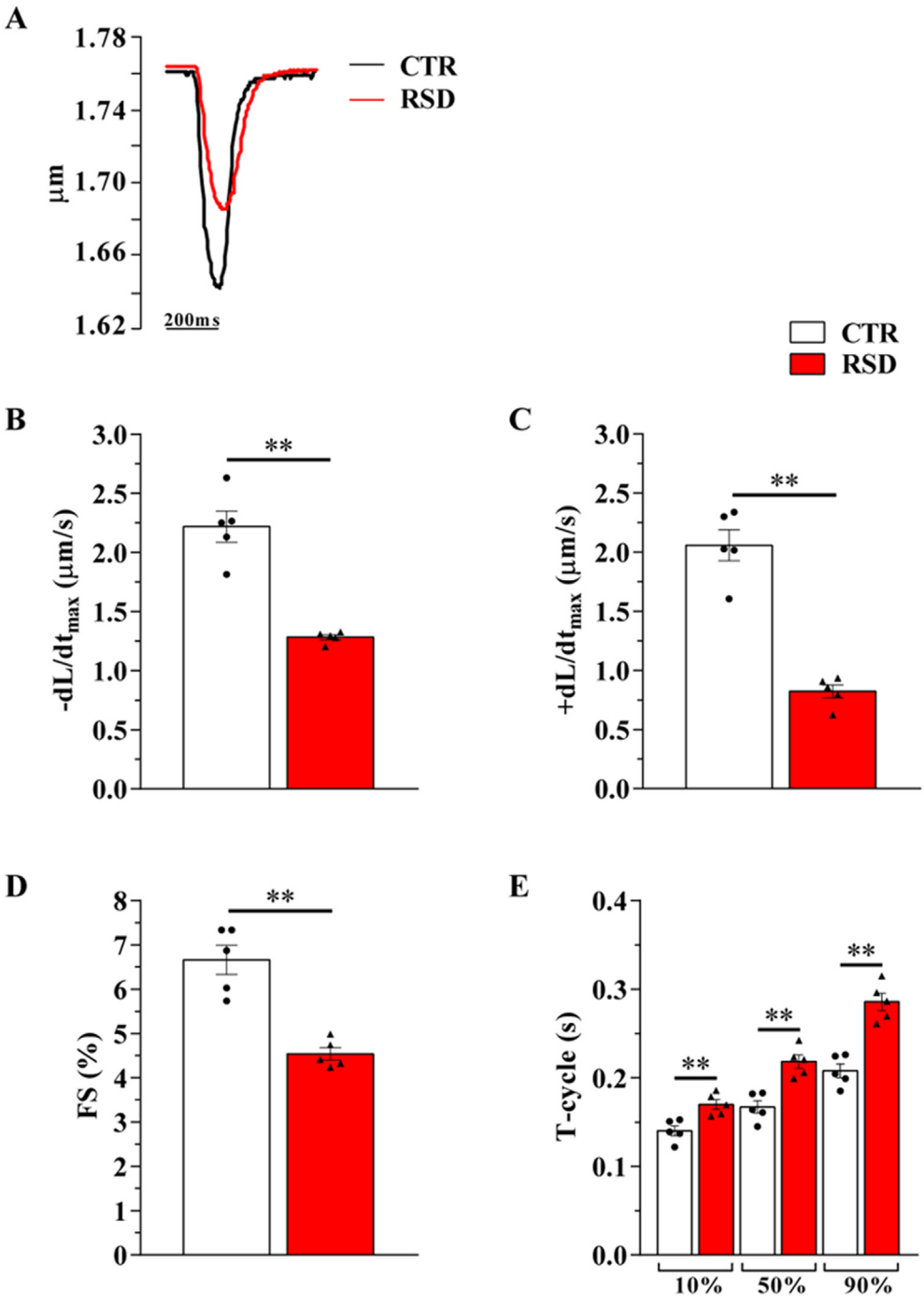

Figure 1. Effects of repeated social defeat stress on cardiomyocyte mechanics. (A) Representative traces of sarcomere shortening in cells obtained from control (CTR) and repeated social defeat (RSD) rats. The histograms (panels B–E) report the maximal rate of shortening (-dl/dt_{max}), maximal rate of relengthening (+dl/dt_{max}), fraction of shortening (FS), and time of total cycle (T-cycle) measured at 10%, 50%, and 90% of relengthening CTR (n = 5; 136 cells) and RSD (n = 5; 127 cells) rats. Data are reported as means ± SEM. **$p < 0.01$.

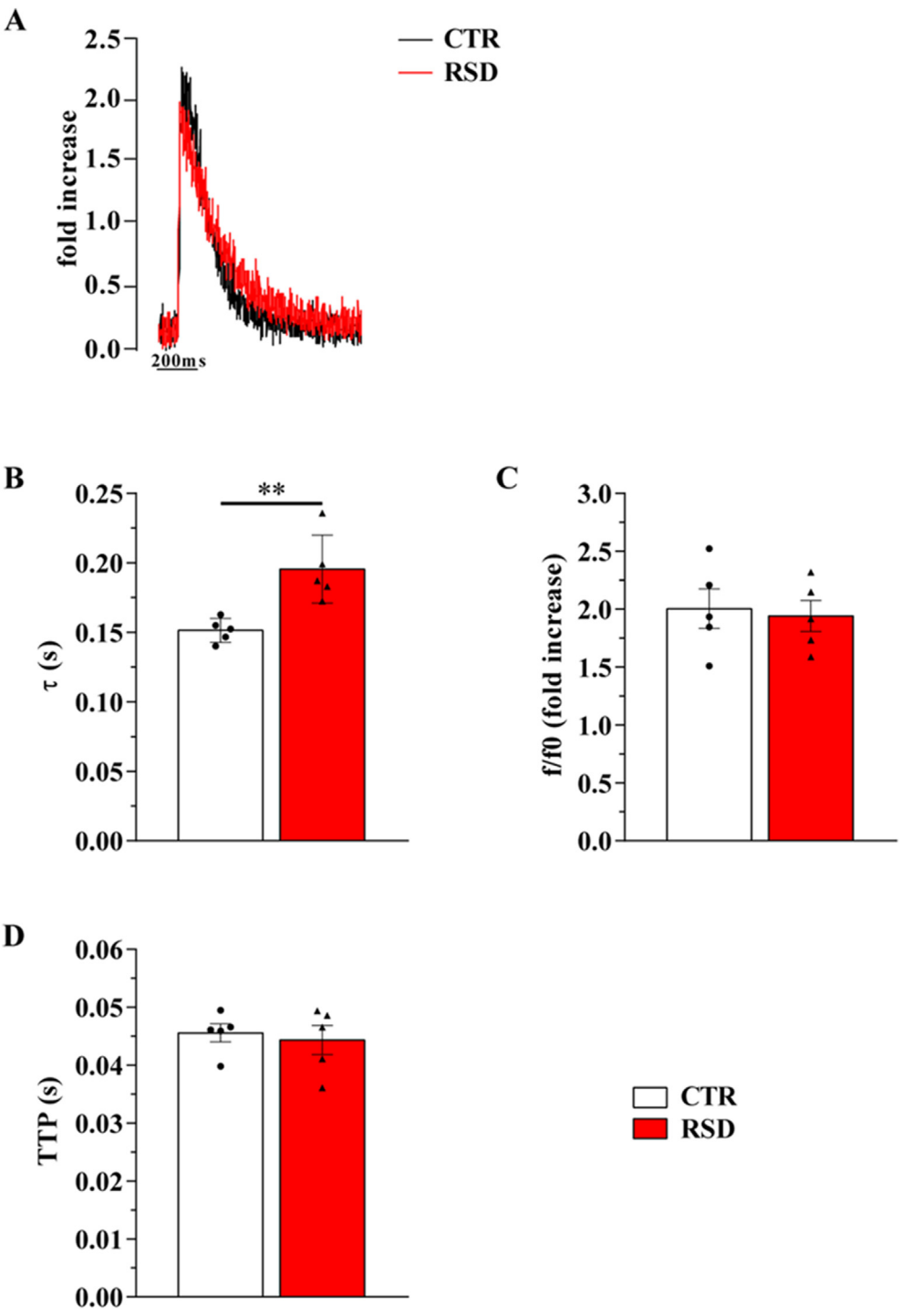

Figure 2. Effects of repeated social defeat stress on calcium transients. (A) Representative traces of calcium transients in cardiomyocytes from control (CTR) and repeated social defeat (RSD) rats. The histograms (panels B–D) report the amplitude of the calcium transient expressed as fluorescence peak normalized to baseline (f/f0), the time constant of intracellular Ca^{2+} decay (τ), and the time-to-peak of the calcium transient (TTP) in CTR (n = 5; 80 cells) and RSD (n = 5; 86 cells) rats. Data are reported as means ± SEM. **p < 0.01.

observed in oxygen consumption rates under coupled (i.e., index of basal respiratory activity) or uncoupled (i.e., index of maximal respiratory activity) conditions (Figure 3A and B). However, the ratio between uncoupled and coupled respiration (i.e., index of normalized reserve respiratory capacity) was significantly lower in RSD cells ($p < 0.05$) (Figure 3C). On the other hand, the difference between uncoupled and coupled respiration (i.e., index of reserve respiratory capacity) was only marginally smaller in RSD cells ($p = 0.08$), which may be ascribed to the presence of an outlier in the RSD group (Figure 3D). In fact, when this value was excluded from the analysis, the difference was significant ($p = 0.016$). Furthermore, ATP content was significantly lower in RSD cells ($p < 0.05$) (Figure 3E). Finally, significant positive correlations were found between indexes of reserve respiratory capacity (i.e., the ratio (Figure 4A) and the difference (Figure 4B) between uncoupled and coupled respiration) and ATP content ($r = .810$, $p < 0.01$ and $r = .733$, $p < 0.05$, respectively).

4. Discussion

The social defeat model is an established and ecologically relevant stress paradigm which induces potent cardiovascular responses in male rats exposed to social subordination by aggressive rats (i.e., social defeat) (Sgoifo et al., 1999, 2014). However, previous studies have generally limited this analysis to the social defeat bout and the immediate

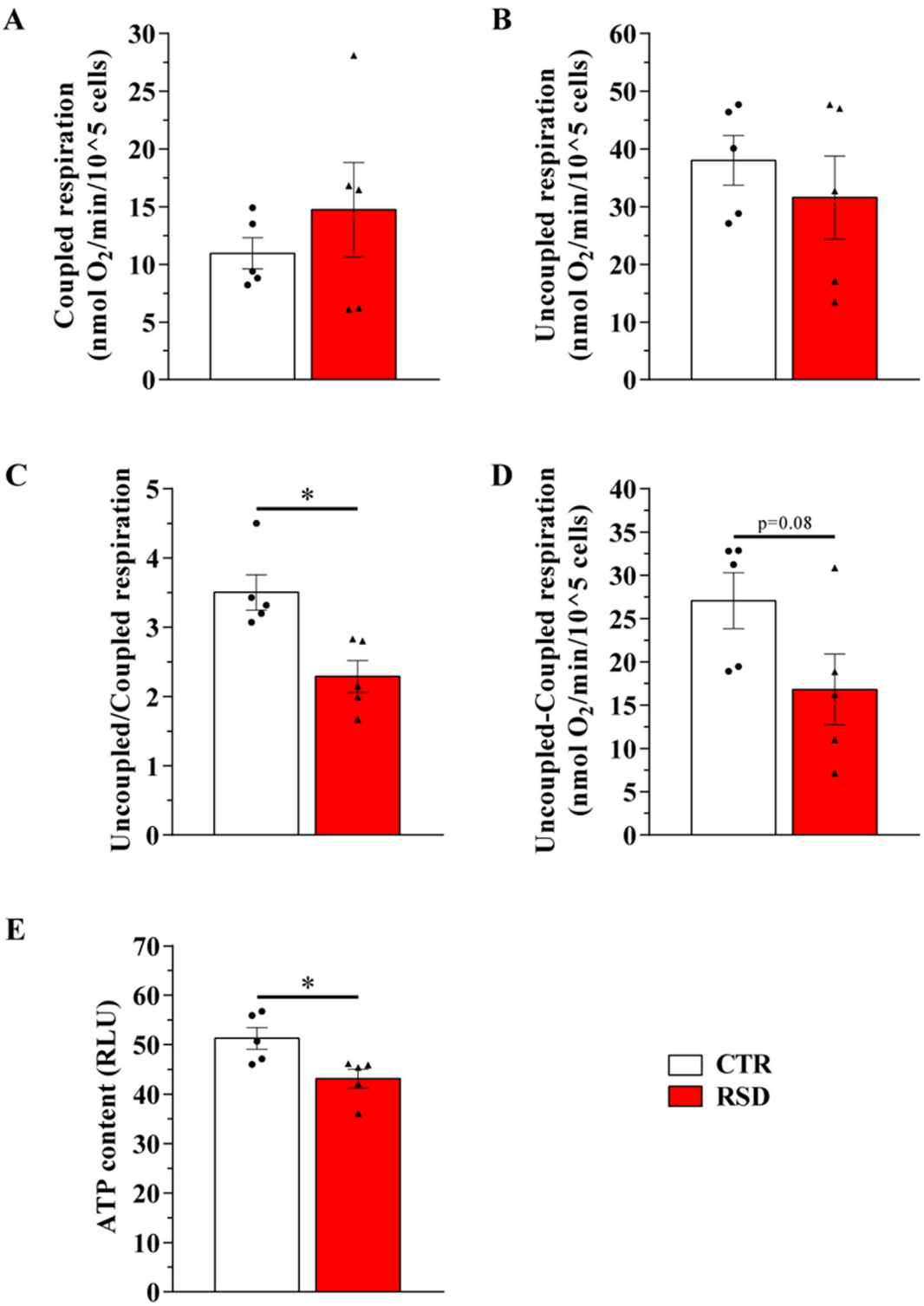

Figure 3. Effects of repeated social defeat stress on mitochondrial respiration and ATP content. The histograms report the basal rate of oxygen consumption (coupled respiration) (A), the maximal rate of oxygen consumption in the presence of the uncoupler Carbonyl cyanide m-chlorophenylhydrazone (CCCP; uncoupled respiration) (B), the ratio between uncoupled and coupled respiration as index of normalized respiratory reserve capacity (C), the difference between uncoupled and coupled respiration as index of respiratory reserve capacity (D), and ATP content (E) in cardiomyocytes from control (CTR) and repeated social defeat (RSD) rats (n = 5 per group). Data are reported as means ± SEM. *p < 0.05.

aftermath (Sgoifo et al., 1999, 2014; Carnevali et al., 2013). Here, we show that stress-induced tachycardic responses were not limited to the 30-min social defeat episode but persisted for several hours after the return of the animals in their home cages following social defeat. These tachycardic responses could not be ascribed to larger baseline levels of somatomotor activity and did not habituate over repeated exposure. Importantly, we describe an increase in baseline HR values over time, which is consistent with previous studies showing alterations in the circadian rhythm of HR after repeated social defeat stress (Sgoifo et al., 1999, 2014; Meerlo et al., 2002). Collectively, these findings are

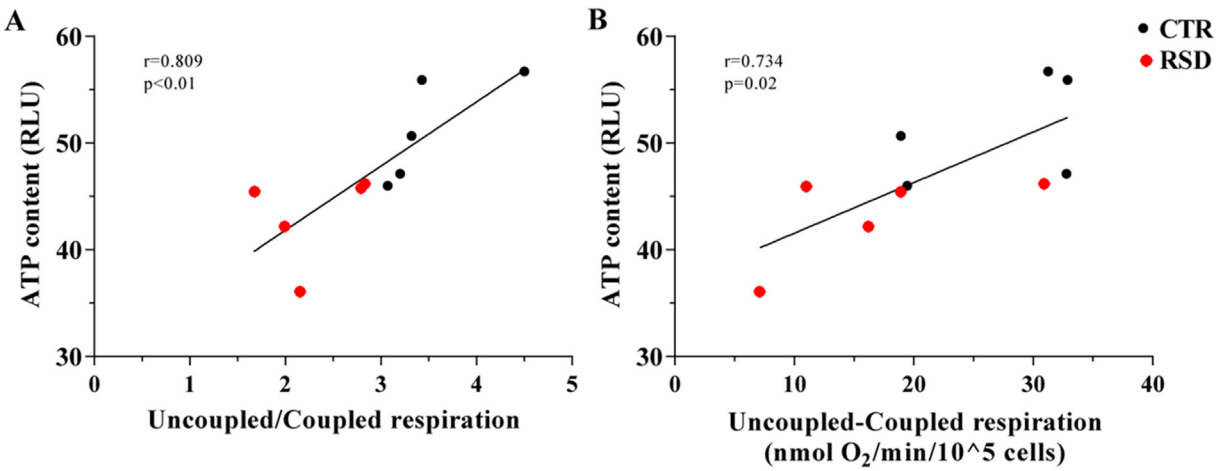

Figure 4. Correlations between indexes of reserve respiratory capacity (the ratio (panel A) and the difference (panel B) between uncoupled and coupled respiration, respectively) and ATP content in CTR (n = 5) and RSD (n = 5) rats.

indicative of prolonged cardiac sympathetic activation in response to repeated social defeat stress, which may lead to cellular and subcellular remodeling of the heart that can be investigated in the context of the pathophysiology underlying social stress-induced CVD risk.

Relatedly, the current results suggest the development of functional decline in the contractile performance of cardiomyocytes isolated from socially stressed rats, as evidenced by lower contraction-relaxation rates and decreased fraction of shortening without corresponding changes in diastolic sarcomere length. Moreover, measurements of intracellular Ca^{2+} dynamics indicated that while intracellular Ca^{2+} rise was unaffected in terms of both amplitude (f/f0) and time to peak, intracellular Ca^{2+} decay (t) was prolonged in cardiomyocytes isolated from socially stressed rats. This finding may reflect abnormalities in cytosolic Ca^{2+} removal by the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) and is consistent with lower relaxation rates in stressed cardiomyocytes. These cellular findings are novel and extend previous works describing the adverse effects of repeated social defeat stress at the organ (heart) level in male rats (Carnevali et al., 2013; Sgoifo et al., 2014; Andolina et al., 2021).

Notably, both cardiomyocyte contractility and SERCA activity strongly depend on ATP availability (Wang et al., 2018). In fact, sarcomeres are composed of overlapping thick and thin filaments and provide the contraction force of the cell through filament displacement, powered by cross-bridge switching by consuming ATP (Skwarek-Maruszewska et al., 2009). Moreover, ATP hydrolysis powers the active transport of Ca^{2+} ions from the cytoplasm to the lumen of the sarco (endo)plasmic reticulum by the intracellular membrane transporter SERCA (Inesi, 1985). Therefore, findings of poor contractile performance and prolonged intracellular Ca^{2+} decay after repeated social defeat may be ascribed to the lower ATP content found in cardiomyocytes of stressed rats.

Our hypothesis is that decreased ATP availability may be due to altered mitochondrial respiratory activity which, in this study, was evaluated by polarographic measurement of oxygen consumption in intact cardiomyocytes. Results indicated that basal (coupled) respiration was not significantly affected by stress exposure. However, basal rates of respiration do not adequately reflect the ability of cellular respiration to respond to conditions of increased energy demand in the contracting heart, such as during overt cardiac sympathetic activation. For this reason, we also measured maximal respiration capacity by adding the uncoupler CCCP. Uncoupled respiration represents the highest respiration rate that cells can reach being free of control by the proton gradient. In particular, the lower ratio and smaller difference between uncoupled and coupled respiration in stressed rats may be interpreted as physiological indicators of reduced reserve respiratory capacity. Furthermore, the strong positive correlations between these parameters and ATP content support the view that reduced respiratory reserve capacity is associated with decreased ATP production.

These findings may be the consequence of mitochondrial uncoupling, a physiological process that has important consequences not only for cellular bioenergetics (i.e., ATP production) but also for reactive oxygen species (ROS) production. It has been suggested that reduced ROS production by mild uncoupling of mitochondrial oxidative phosphorylation may represent a cytoprotective strategy under conditions of oxidative stress such as diabetes, ischemia-reperfusion injury, or aging (Cadenas, 2018). Interestingly, a previous study documented the presence of oxidative stress in cardiomyocytes of rats exposed to a chronic social stress paradigm based on the visible burrow system model (Turdi et al., 2012). Therefore, partial mitochondrial uncoupling may represent an attempt to limit ROS production and attenuate oxidative damage and cell death in cardiomyocytes under conditions of repeated social defeat stress. However, as discussed above, the limited availability of ATP compromises cardiomyocyte contractile performance and calcium clearing, leading to a vicious circle that may ultimately lead to pathological processes. In fact, severe uncoupling has been associated with mitochondrial depolarization and exacerbated injury in rat ventricular myocytes (Brennan et al., 2006). Nevertheless, these mechanistic hypotheses need to be experimentally tested, for example by using oligomycin to examine proton leak or indicators of mitochondrial membrane potential. In fact, we cannot rule out the possibility that reduced ATP content in cardiomyocytes of socially stressed rats may not just be due to uncoupling but may be a direct result of altered ROS production (Manoj, 2018). Furthermore, it must be noted that the activation of inflammatory factors has been implicated in social defeat stress-induced myocardial injury in mice (Gao et al., 2020) as well as in mitochondrial dysfunction and altered bioenergetics (Trinchese et al., 2021; Billingham et al., 2022), suggesting a role of inflammatory processes in social stress-induced cardiomyocyte and mitochondrial changes.

4.1. Conclusions and perspectives

Mitochondrial dysfunction has been extensively implicated in CVD (e.g., Chistiakov et al., 2018). Quite surprisingly, despite chronic social stress represents a well-recognized risk factor for CVD, the study of the association between cardiac and mitochondrial functional decline under stressful conditions has largely been neglected by preclinical stress research. The current investigation represents a first attempt to fill this substantial knowledge gap. Here, we show that male rats exposed to repeated social stress develop cellular mechanical dysfunctions and intracellular Ca^{2+} derangement associated with mitochondrial changes

that may represent the transition point from an adaptive stress response to the beginning of a functional decline that ultimately affects cardiomyocyte health.

However, it must be acknowledged that these results should be interpreted within the context of their limitations. First, this study provides indirect correlations between cardiac and mitochondrial functional data and does not investigate the events in a dependent (causal) manner. Second, analysis of mitochondrial function has been limited to simple measures of respiration and ATP production and future experiments implementing other measurements (e.g., mitochondrial membrane potential, ROS production) are needed to corroborate our hypotheses. Third, we do not provide any behavioral or biochemical (e.g., corticosterone levels) measure to verify whether individual differences in coping responses to social stress are associated with the magnitude of cardiomyocyte contractile/bioenergetic defects. Fourth, analyses were conducted 24 h after the last social stress exposure and cannot indicate the extent to which the described changes are reversible or enduring. Lastly, these male-based findings cannot be generalized to females, particularly because male and female organisms have qualitatively different mitochondria and little is known about their interaction with stress (Ventura-Clapier et al., 2017).

In conclusion, this study suggests that repeated social stress impairs contraction/relaxation properties and intracellular calcium dynamics of the male rat heart and points to mitochondrial dysfunction as a putative pathophysiological process underlying increased CVD risk. Future studies adopting longer social stress protocols, multiple and deeper assessments of mitochondrial function and, preferably, animals of both sexes are needed to describe the trajectory of mitochondrial functional decline under condition of chronic stress, and its consequences on cardiac health. These studies will likely shed new light on the pathophysiological processes leading to stress-related CVD and inspire the development of innovative preventive strategies. Among these, approaches that target epigenetic mediators which are sensible to stress and regulate mitochondrial dynamics (e.g., microRNA-34a (Andolina et al., 2021)) might be promising.

Declarations

Author contribution statement

Margherita Barbetti; Rocchina Vilella: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Cristina Dallabona: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Maria Carla Gerra; Leonardo Bocchi; Donald Ielpo: Analyzed and interpreted the data.

Diego Andolina: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Andrea Sgoifo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Monia Savi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Luca Carnevali: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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