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Sex differences in cardiac mitochondrial function following oxygen-glucose deprivation in primary cardiomyocytes

Erica Underwood
The University of Texas Health Science Center at Houston
Andrea Becerra Calixto
The University of Texas Health Science Center at Houston
Theodore Gallas
The University of Texas Health Science Center at Houston
Brenna Servantes
The University of Texas Health Science Center at Houston
Matthew DeBerge
The University of Texas Health Science Center at Houston
Louise D. McCullough
The University of Texas Health Science Center at Houston
Tina O. Findley
Tina.O.Findley@uth.tmc.edu
The University of Texas Health Science Center at Houston

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Abstract

Metabolic adaptation to hypoxic-ischemic stress is cardioprotective; however, sex-specific differences in mitochondrial function following oxygen-glucose deprivation and reperfusion (OGD/R) remain poorly defined. This study examined whether cardiomyocytes exhibit differential mitochondrial responses to acute and prolonged OGD/R. Primary human cardiomyocytes derived from age-matched male and post-menopausal female donors were exposed to OGD/R for 30 minutes (acute), 5 hours (prolonged), or control conditions. Mitochondrial function was assessed using Seahorse extracellular flux analysis, measuring oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and respirometry parameters. Following acute OGD/R, male cardiomyocytes exhibited reduced maximal respiration, whereas female cardiomyocytes maintained maximal respiratory capacity. In contrast, after prolonged OGD/R, female cardiomyocytes demonstrated reduced maximal respiration, while male cardiomyocytes recovered OCR to levels comparable to controls. At baseline, female cardiomyocytes showed higher ECAR, consistent with greater reliance on glycolysis. This glycolytic advantage was lost after prolonged OGD/R. These findings demonstrate temporally distinct, sex-associated responses to OGD/R. Female cardiomyocytes exhibit early mitochondrial engagement followed by reduced oxidative recovery under sustained stress, whereas male cardiomyocytes maintain coordinated glycolytic and oxidative activity. These data suggest that sex differences in mitochondrial adaptability may contribute to differences in susceptibility to hypoxic-ischemic injury, underscoring the importance of incorporating biological sex into metabolic studies.

Introduction

Cardiovascular disease is the leading cause of death in women worldwide.[1] In the United States, heart disease accounts for one in three female deaths.[2] The incidence of heart disease significantly increases after menopause due to the loss of the cardioprotective effects of estrogen.[3] Post-menopausal women have a disproportionately higher risk of heart failure and death after acute myocardial ischemic events and coronary artery surgery compared to men.[4–9] Irreversible myocardial injury is a key contributor to cardiac dysfunction and is associated with increased morbidity and mortality. Given the high metabolic demands of the heart and its reliance on mitochondrial respiration for approximately 90% of its energy supply, impairment of mitochondrial function results in cardiac dysfunction and heart failure. Sex differences in mitochondrial bioenergetics may play a pivotal role in the increased mortality observed in aged females.[10–13]

Sex- and age-dependent differences in cardiac mitochondrial function have been demonstrated in animal models. Aged female cardiomyocytes exhibit a more significant reduction in mitochondrial enzymatic activity than aged males.[14] At the transcriptional level, cardiac mitochondrial function is sexually dimorphic, with adult-aged female rats demonstrating a greater reliance on oxidative phosphorylation in the absence of disease.[15,16] In the setting of hypoxic-ischemic injury, such as myocardial infarction and heart failure, a compensatory metabolic shift favoring glycolysis occurs, which has sparked interest in

targeting cardiac metabolism to mitigate heart failure.[17–19] However, whether this metabolic shift is sexually dimorphic is not well understood in studies that do not account for sex as a biological variable, particularly in cell culture experiments, preventing the discovery of critical targeted sex-specific therapeutics.[20–22] Furthermore, traditional mitochondrial assays used to measure mitochondrial function require cell lysis or endpoint measurements, therefore lacking real-time, dynamic assessment of mitochondrial function and stress in live cells. In this study, we use oxygen-glucose deprivation with reoxygenation (OGD/R) as an *in vitro* model of hypoxic-ischemic injury to study its effects on cardiac mitochondrial function using sex-differentiated primary human cardiomyocytes. We also characterized the metabolic consequences of two durations of OGD on mitochondrial function by Seahorse assay.

Materials and Methods

Primary human ventricular cardiomyocytes isolated from an adult male donor (52 years old; Lot 9) and a female donor (53 years old; Lot 13) were obtained commercially from Creative Bioarray (Shirley, NY; Cat. No. CSC-C2847). Donor sex was reported by the vendor. Cells were thawed and handled according to the manufacturer's instructions, then seeded onto plates pre-coated with 0.1% gelatin for expansion. The culture complete medium consisted of DMEM supplemented with L-glutamine, D-glucose, 10% fetal bovine serum (Corning, Incorporated, NY, USA), 1% B27 supplement (Gibco, Thermo Fisher Scientific, Waltham, MA), and a penicillin-streptomycin-amphotericin B cocktail (Gibco, Thermo Fisher Scientific, Waltham, MA). Cardiomyocyte identity and purity were verified by the manufacturer by flow cytometry for cardiac troponin I (TNNI3), and sarcomeric organization was confirmed by immunofluorescent labeling for cardiac alpha-actin with confocal laser scanning microscopy (vendor certificate of analysis; Creative Bioarray).

Twenty-four hours before oxygen-glucose deprivation and reoxygenation (OGD/R) exposure, cardiomyocytes were seeded into XF Pro Cell Culture Microplates (Agilent Technologies, Inc., Santa Clara, CA) at different densities of 6×10^3, 9×10^3, and 12×10^3 cells/well. The plates were then incubated at 37°C in complete growth medium to allow for cell attachment and stabilization before the onset of the experiment.

OGD Exposure

Prior to OGD/R, cells were washed with PBS without Calcium and Magnesium twice and incubated in DMEM glucose and sodium pyruvate-free (Gibco, Thermo Fisher Scientific, Waltham, MA), serum-free medium, and pre-equilibrated to hypoxic conditions. Plates were transferred to a hypoxia chamber (STEMCELL Technologies, Vancouver, Canada) and maintained at 37ºC under low oxygen (1% O_2, 5% CO_2, balance N_2). Cells were exposed to OGD for 30 minutes (modeling acute ischemic/metabolic stress) or 5 hours (modeling prolonged ischemic/metabolic stress). Following OGD, reperfusion was modeled by reoxygenation: OGD medium was replaced with prewarmed complete culture medium and plates were returned to normoxic conditions (21% O_2, 5% CO_2, 37ºC) for 1 hour. Time-matched normoxic

controls were maintained in parallel in glucose-containing medium under normoxia for the corresponding durations. At the end of reoxygenation, cells were processed immediately for the Seahorse assay.

Mitochondrial Bioenergetic Response

A modified Agilent Mito Stress assay was used to assess mitochondrial function in cultured cardiomyocytes by quantifying real-time oxygen consumption rate (OCR, pmol/min), as an index of oxidative phosphorylation. Extracellular acidification rate (ECAR, mpH/min) was measured concurrently as an indicator of glycolytic flux, enabling estimation of the relative contributions of oxidative phosphorylation and glycolysis to ATP production. The assay consisted of three replicates per condition with each OGD condition (normoxic, 30 min, and 5 hours) on an individual plate. One hour prior to respirometry, the media present during OGD were removed, the cells gently washed twice, and the media replaced with freshly made assay media. The assay media consisted of XF Base Medium (Agilent, 103335; pH 7.4, without phenol red) plus mitochondrial substrates: pyruvate (1 mM), glutamine (2mM), and glucose (5 mM). To perform a modified Cell Mito Stress Test (Agilent, 103015) the injection ports of a pre-calibrated cartridge were loaded with optimized concentrations of the following inhibitors: port A: oligomycin (1μM final); port B: FCCP (0.75μM final); and port C: Rotenone/Antimycin A (0.5μM/0.5μM final) + 2-deoxy-D-glucose (50mM, Sigma D8375). All assays were performed using phenol red-free assay medium to avoid potential estrogen receptor-mediated effects of phenol red and reduce confounding of sex-specific responses.

Statistical analyses were performed in GraphPad Prism 10. OCAR and ECAR time-course data were analyzed using two-way ANOVA model or a mixed effects model if values were missing with Dunnett's multiple comparisons test. Each respirometry parameter was analyzed using two-way ANOVA with factors of OGD duration, sex, and sex x duration interaction. Post-hoc Tukey's multiple comparisons tests were used to test prespecified effects of OGD duration within each sex and between sexes for each OGD duration. Statistical significance was defined as an adjusted p-value < 0.05.

Results

To examine the sex-associated effects of hypoxic-ischemic injury and reoxygenation on cellular metabolism, we measured OCR and ECAR in primary cardiomyocytes following OGD/R. A representative OCR curve showing the expected responses to various inhibitor injections used to interrogate different aspects of mitochondrial respiration is shown in Fig. 1A. Basal OCR was determined by measuring respiration before any inhibitor injection. Oligomycin, an adenosine triphosphate (ATP) synthase inhibitor, blocks proton flux through ATP synthase (Complex V), reducing OCR to reveal respiration linked to ATP synthesis. The residual proton flux and related oxygen consumption after oligomycin addition to the reaction represent a measure of proton leak. The uncoupler FCCP, which abolishes the proton gradient, results in maximal OCR. Finally, the addition of rotenone, a Complex I inhibitor, and antimycin A, an

inhibitor of cytochrome C reductase, completely abolishes mitochondrial-mediated oxygen respiration. Any remaining residual oxygen consumption after the addition of rotenone/antimycin A can be attributed to nonmitochondrial oxygen-consuming enzymes in the sample, such as oxidases. To allow simultaneous analysis of glycolysis during these assays, 2-deoxyglucose was added to the final rotenone/antiA injection to competitively inhibit the production of glucose-6-phosphate from glucose at the phosphoglucoisomerase step (step 2 of glycolysis).[23]

Our results demonstrated that cardiomyocytes in the absence and presence of hypoxic-ischemic injury exhibit a sexually dimorphic bioenergetic response. Under normoxic conditions, overall OCR and ECAR activity were significantly higher in female cardiomyocytes than in males (OCR mean difference of 2.09 pmol/min, p = 0.0266; ECAR mean difference of 1.99 mpH/min, p = 0.0002). In both sexes, OCR was significantly reduced after acute (30 minutes) OGD (female, p < 0.0237; male, p < 0.0001) (Table 1). In contrast, after prolonged (5 hours) OGD, OCR activity was similar to that of controls (Fig. 1). Overall ECAR activity following acute OGD was similar to controls in both sexes; however, in female cardiomyocytes only, ECAR was reduced following prolonged OGD (p = 0.0186) (Fig. 2).

Table 1
Comparisons of OCR and ECAR within each sex following 30-minute or 5-hour OGD/R (mixed effects analysis). P-values were adjusted for Dunnett's multiple comparisons, with a significance threshold of adjusted p < 0.05.

	Female				**Male**			
	OCR (pmol/min)		**ECAR (mpH/min)**		**OCR (pmol/min)**		**ECAR (mpH/min)**	
	Mean diff (95% CI)	p_{adj}-val	Mean diff (95% CI)	p_{adj}-val	Mean diff (95% CI)	p_{adj}-val	Mean diff (95% CI)	p_{adj}-val
30 min OGD/R	-4.88 (-8.92, -0.84)	**0.0237**	0.39 (-1.50, 2.27)	0.7869	-5.51 (-7.17, -3.85)	**0.0001**	-0.45 (-1.39, 0.49)	0.4085
5 hour OGD/R	-0.18 (-4.22, 3.86)	0.9885	-2.42 (-4.30, -0.53)	**0.0185**	1.67 (-0.04, 3.29)	0.0548	-0.84 (-1.78, 0.09)	0.0737

OCR, oxygen consumption rate; ECAR, extracellular acidification rate.

Respirometry parameters were compared between sexes to determine which component of mitochondrial function was most affected by sex. Basal respiration was similar between and within each sex group as expected (Fig. 3A). The lack of change in basal respiration after OGD suggested no significant cell loss due to hypoxia-induced cell death. Therefore, the raw respiratory values, normalized to an initial plating density, were compared with other respiratory parameters without post-hypoxia normalization. Acute OGD caused a significant reduction in maximal respiration in male cardiomyocytes

(mean difference of -4.18 pmol/min, p = 0.0006) with minimal effect in female cardiomyocytes, whereas prolonged OGD impaired maximal respiration in female cardiomyocytes (mean difference of -3.78 pmol/min, p = 0.0013) with no effect in males (Fig. 3B). ATP-linked respiration was most impacted by prolonged OGD in female cells only (mean difference of -2.88 pmol/min, p = 0.0156) (Fig. 3C). Proton leak and spare capacity were similar across both durations of OGD in both sex groups (Fig. 3D-E). Non-mitochondrial respiration was significantly lower in both sexes following acute OGD (male mean difference of -3.99 pmol/min, p = 0.0088; female mean difference of -3.86 pmol/min, p = 0.0110) but was similar to controls in the setting of prolonged OGD (Fig. 3F). This indicates a transient reduction in oxygen-consuming oxidase activity, consistent with a global suppression of oxygen-consuming pathways under acute stress. In prolonged OGD, non-mitochondrial respiration returned to levels comparable to controls, maintaining activation or adaptation of non-mitochondrial oxygen-consuming processes.

Energy map analysis revealed a marked difference in metabolic shift following OGD exposure (Fig. 4). OCR/ECAR ratio serves as a measure of overall metabolic preference and energetic state. Male cardiomyocytes clustered predominantly in the quiescent/low-energy region (represented by a low OCR/low ECAR ratio) following acute OGD injury, consistent with a metabolically suppressed state. In contrast, female cardiomyocytes remained in the energetic quadrant (high OCR/high ECAR) following acute OGD, using both metabolic pathways robustly despite injury. When OGD exposure became prolonged, both sexes increased their aerobic activity, reflecting a decreased reliance on glycolysis (high OCR/low ECAR).

Discussion

Ischemic heart disease is the leading cause of death worldwide,[24] and a better understanding of contributors to sexually dimorphic morbidity and mortality rates poses an opportunity for improving clinical outcomes.[10] More than 90% of myocardial ATP is produced by mitochondrial oxidative phosphorylation; therefore, the heart relies heavily on the mitochondrial system.[25,26] Heart failure is a well-known consequence of cardiometabolic dysfunction,[17,27] and sex-specific differences in outcomes after myocardial ischemic injury have been linked to impaired mitochondrial function.[28–30] Mitochondrial respiration activity has been shown to be higher in females than in males,[16,31] indicating the possibility of an inherent sex bias in cellular metabolism. Prior studies have identified key metabolic disruptions associated with ischemic-reperfusion injury, including impaired oxidative phosphorylation, increased reactive oxygen species (ROS) production, and mitochondrial permeability transition.[32–35] Moreover, age- and sex-specific differences in the ability to shift substrate utilization preferentially during these disruptions, thereby altering mitochondrial efficiency under stress, suggest a sexually dimorphic capacity for metabolic adaptation.[11,14,36] Among these shifts, glycolytic metabolism has garnered attention as a potentially manipulable pathway in heart failure.[25] However, whether male and female cardiomyocytes engage glycolytic pathways differently during ischemia remains unclear. To better understand whether this mechanism contributes to sex-based disparities in ischemic heart disease, this study investigates

mitochondrial metabolic responses in primary human ventricular cardiomyocytes from age-matched male and postmenopausal female subjects during OGD followed by reoxygenation.

While basal mitochondrial respiration levels were similar between male- and female-derived cardiomyocytes, acute and prolonged OGD/R induced sexually dimorphic changes in maximal respiration activity. Male cardiomyocytes exhibited lower maximal respiration following acute (30 minutes) OGD/R, while no difference was observed in female cardiomyocytes. However, female cardiomyocytes displayed significantly lower maximal respiration following prolonged (5 hours) OGD/R, whereas male cardiomyocytes recovered OCR to levels comparable to those of controls. The observed differences in ATP-linked respiration further suggested that females employ adaptive mechanisms under acute metabolic stress. Female cardiomyocytes appeared to mount an early, compensatory increase in ATP-linked oxidative phosphorylation that was not sustained under prolonged metabolic challenge; however, male cardiomyocytes maintained a stable profile of ATP production across all conditions. This pattern revealed that female cardiomyocytes initially engage greater mitochondrial responsiveness to meet acute energetic demand, but at the cost of earlier mitochondrial exhaustion during extended OGD/R, while males exhibit a more conservative respiratory output that may reflect reduced metabolic flexibility but greater resistance to acute mitochondrial overactivation. Together, these findings support the presence of sex-associated differences in the mitochondrial system and stress adaptation during ischemic injury.

The changes observed in non-mitochondrial respiration demonstrated that redox-related oxygen consumption was differentially regulated during acute versus chronic metabolic stress. The reduction in non-mitochondrial respiration following acute OGD/R likely reflected transient suppression of oxygen-consuming oxidases, such as NADPH oxidases and other ROS-generating enzymes.[37] This response may represent an early protective mechanism that limits oxidative damage during a sudden energy crisis. In contrast, the return of non-mitochondrial respiration to baseline levels with chronic stress may reflect reactivation of oxidase activity as cells transition to a state of sustained stress adaptation. These findings indicated that cardiomyocyte responses to metabolic stress involved temporally distinct redox adaptations. This uniform pattern across both sexes also revealed that while mitochondrial responses to OGD/R were sex-associated, non-mitochondrial responses may follow a common trajectory.

Differences in ECAR further supported a baseline metabolic divergence triggered by ischemic-reperfusion injury. Under normoxic conditions, female cardiomyocytes showed greater reliance on or capacity for glycolysis, consistent with metabolic flexibility observed in the energy map analysis. In acute hypoxic stress, glycolytic activity was similar to controls in both sexes. However, after prolonged OGD/R, the selective reduction of ECAR in female cardiomyocytes implied a loss of this glycolytic advantage, potentially reflecting substrate depletion, impaired glycolytic enzyme activity, or a metabolic shift as a protective mechanism. This response in ECAR was accompanied by reduced ATP-linked OCR despite preserved spare capacity, suggesting that mitochondrial capacity remained intact in female cardiomyocytes but was not fully utilized for ATP synthesis under prolonged stress. In contrast, glycolytic activity was sustained during prolonged stress in male cardiomyocytes with preserved ATP-

linked respiration and spare respiratory capacity, both indicators of downstream oxidative recovery, implying coordinated engagement of both glycolytic and oxidative pathways. This pattern was consistent with effective metabolic compensation. These findings demonstrate that male cardiomyocytes sustain active energy production during prolonged stress, whereas female cardiomyocytes transition to a lower-energy state while preserving mitochondrial reserve. One possible mechanism is the previously described sex-dependent transcriptional regulation of oxidative metabolism,[16,30] including pathways governed by PPARα and other related nuclear receptors that promote fatty acid oxidation and mitochondrial maintenance.[38–40] Enhanced activation of these pathways in females could preserve mitochondrial capacity while limiting glycolytic flux and overall energy turnover during sustained stress. Further studies examining this framework will clarify drivers of sex-specific differences in transcriptional control of metabolic pathways.

This study has several limitations. First, the use of cardiomyocytes from postmenopausal women does not fully capture sex differences across the lifespan. Loss of circulating estrogen and age-associated mitochondrial changes represent a phenotype not observed in younger myocardium. Therefore, the findings should be interpreted as reflecting sex differences within an aging context rather than intrinsic biological sex effects alone. Second, bioenergetic measurements were obtained using the Seahorse assay, which provides indirect estimates of glycolytic and oxidative metabolism based on oxygen and proton production. While powerful, this platform does not directly measure substrate flux, ATP production, or redox state. Additionally, this study used primary cardiomyocytes from a single donor of each sex. The observed differences should therefore be interpreted as sex-associated as other donor-specific factors cannot be separated from sex. However, donor-matched pair offered a controlled starting point for testing whether metabolic responses differ by sex under identical culture and treatment conditions. Replicates were independent cultures from each donor, capturing experimental reproducibility but not variation between individuals within each sex. Lastly, the OGD/R model represents *in vitro* ischemia-reperfusion injury and thus cannot replicate the complex *in vivo* environment, including hormonal signaling and mechanical load. These limitations highlight the need for complementary approaches to extend these findings.

In summary, these findings reveal sex-associated differences in cardiomyocyte metabolic adaptation to hypoxic stress that depend on the duration of injury. Our study uncovers distinct bioenergetic responses during prolonged injury, with female cardiomyocytes favoring metabolic downregulation and mitochondrial preservation, while male cardiomyocytes maintain active energy production. This may reflect fundamentally different approaches to balancing energy demand during stress. These results highlight sex as a possible determinant of cardiomyocyte metabolic response to hypoxic stress and underscore the need to incorporate sex-specific targeted therapies.

Declarations

Funding: No external funding was received for this study.

Author contributions

Conceptualization: Tina O. Findley, Louise D. McCullough

Formal analysis: Erica Underwood

Investigation: Erica Underwood, Theodore Gallas, Brenna Servantes

Methodology: Erica Underwood, Andrea Becerra Calixto

Supervision: Erica Underwood, Tina O. Findley

Writing – original draft: Tina O. Findley

Writing – review & editing: Erica Underwood, Andrea Becerra Calixto, Matthew DeBerge, Louise D. McCullough, Tina O. Findley

Data availability statement: All relevant data are within the manuscript.

Competing Interests statement: The authors have declared that no competing interests exist.

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Figures

Figure 1.

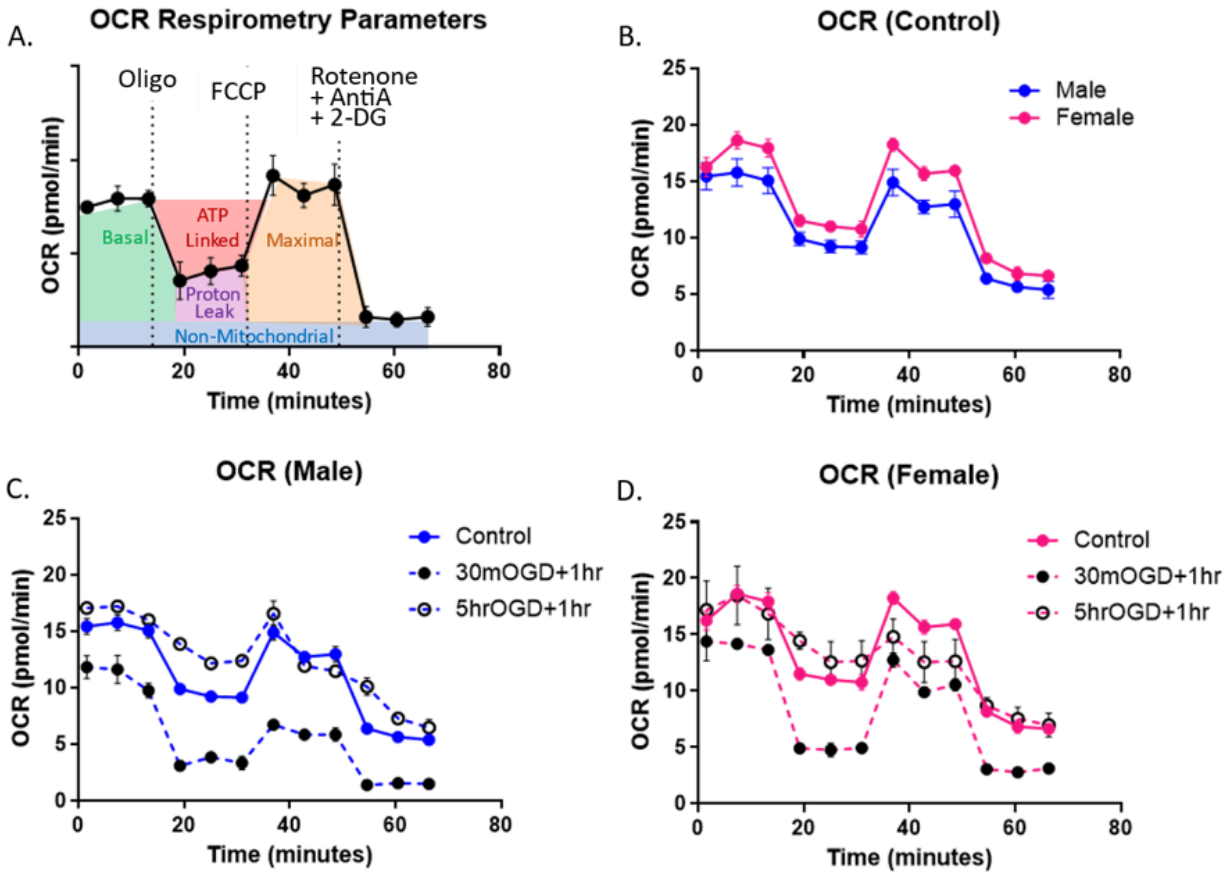

Figure 1

Seahorse assay of primary cardiomyocytes from male and female donors following OGD. A) A representative schematic of expected respirometry parameters. B) Overall OCR activity was higher in female cardiomyocytes in normoxic conditions ($p = 0.0266$). Mitochondrial responses in C) male and D) female cardiomyocytes following acute (30 minutes) and prolonged (5 hours) OGD/R. Data analyzed by two-way ANOVA or mixed effect analysis, Dunnett's multiple comparisons test.

Figure 2.

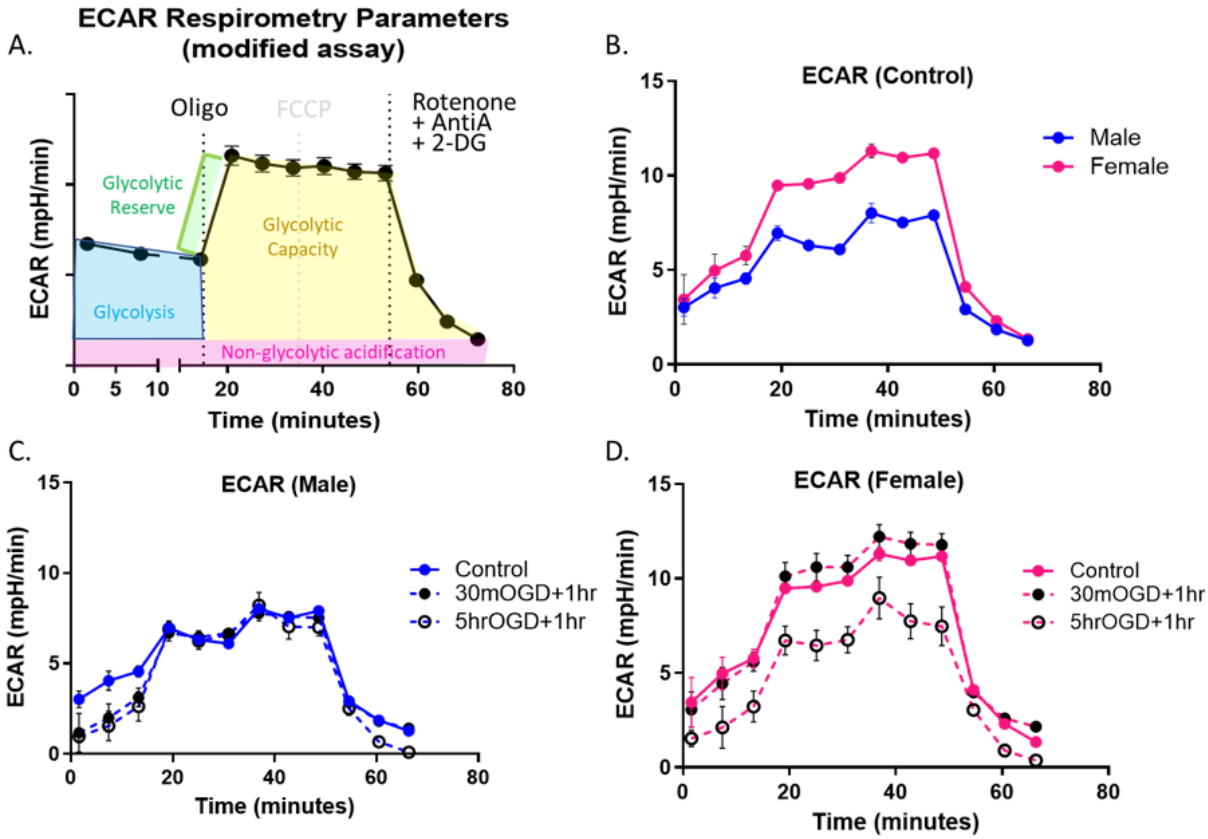

Figure 2

ECAR analysis in primary cardiomyocytes from male (blue) and female cardiomyocytes (pink) following OGD exposure.

Figure 3.

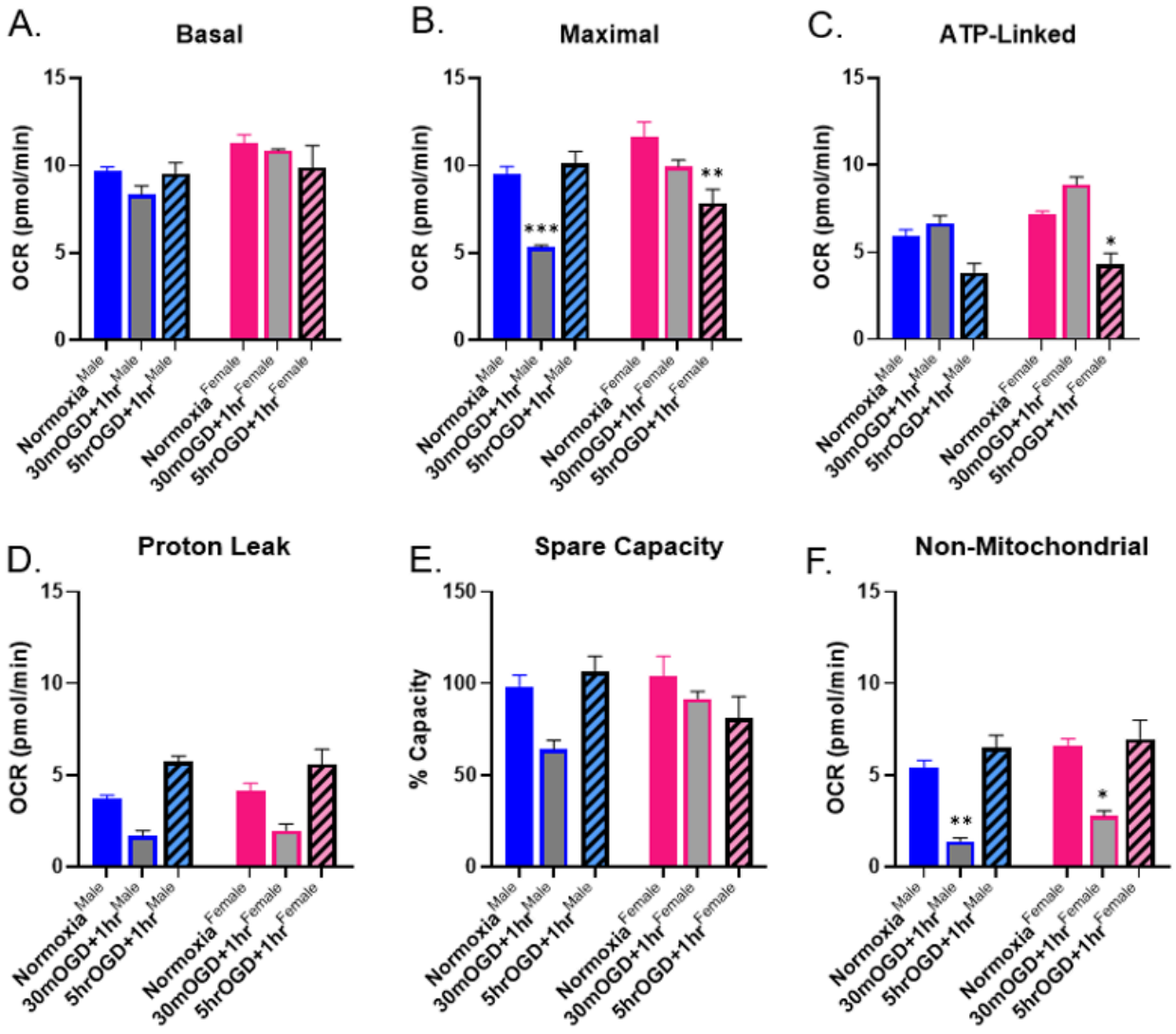

Figure 3

Respiratory parameters of Seahorse assay comparing responses of male and female cardiomyocytes follow two durations of OGD. Data analyzed by two-way ANOVA, Tukey's multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001).

Figure 4.

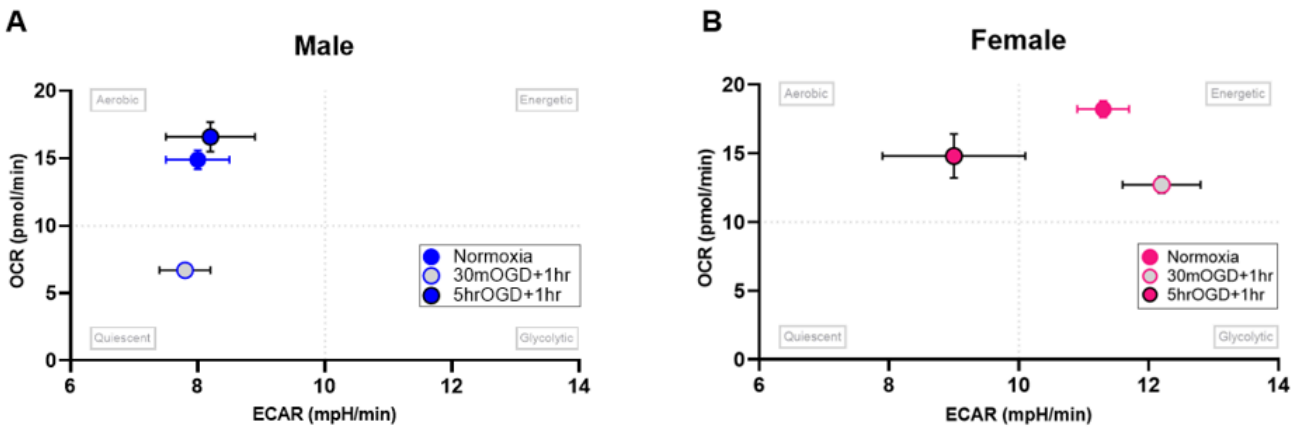

Figure 4

Seahorse energy map analysis (OCR vs. ECAR) in primary cardiomyocytes from male and female donors following OGD exposure.