Supplementary Materials for:

GLUT1 Deficiency in Cardiomyocytes Does not Accelerate the Transition from Compensated Hypertrophy to Heart Failure

Running title: ***Pereira et al.; GLUT1 deficiency in POH***

Renata O. Pereira, Adam R. Wende , Curtis Olsen , Jamie Soto , Tenley Rawlings , Yi Zhu , Christian Riehle, and E. Dale Abel

1. DETAILED METHODS:

1.1. Composition of mouse chow

Mice were fed standard chow, Harland Teklad Diet 8656 (3.8 Kcal/g of gross energy) that contained 65% carbohydrate (corn and soybean meal), 24.5 % protein (Soy based), 4.4 % fat (Soybean oil), 3.4 % fiber and was supplemented with vitamins and minerals.

1.2. Isolation of cardiac myocytes and determination of 2-DG uptake

Cardiomyocytes were isolated using collagenase digestion of Langendorff-perfused control and G1KO hearts as previously described by our group [1]. This isolation procedure initially yields between 80 and 90% viable rod-shaped myocytes, the majority of which attach to laminin-coated wells and maintain their morphology for the duration of the protocol. Basal glucose uptake was measured using 2-deoxyglucose. In brief, cardiomyocytes were prepared from mouse hearts that were obtained from heparinized mice and retrograde perfused with perfusion buffer (pH 7.3; in mmol/l): 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 4.0 $NaHCO_3$, 10.0 HEPES, 30.0 2,3-butanedione monoxime, 5.5 glucose, 1.8 pyruvate, and 0.025 $CaCl_2$ supplemented with 16 U/ml type I collagenase for 10 min. Myocytes were dispersed for 10 min at 37°C in perfusion buffer that contained 2% BSA and 0.2 mmol/l $CaCl_2$ and cultured in modified Dulbecco's modified Eagle's medium (DMEM) that contained 5% FBS, 5.0 mmol/l glucose, and 1.0 mmol/l $CaCl_2$ on laminin-coated plates at 37°C in humidified 95% O_2–5% CO_2. Cells were allowed to attach to the wells for 60 min and were subsequently cultured for 30 min in DMEM that contained 0.1% BSA, 5.0 mmol/l glucose, and 1.0 mmol/l $CaCl_2$ before measuring glucose uptake. Cells were then incubated for 40 min, in glucose-free DMEM supplemented with 1 mg/ml BSA and 1 mmol/l pyruvate. Glucose uptake was performed by adding 0.1 mmol/l 2-deoxy-d-glucose and 3.33 nCi/ml 2-[1,2-^{3}H]-deoxy-d-glucose for 30 min. Glucose transport experiments were terminated after 30 min by aspiration of the buffer followed

by two washes with ice-cold PBS and then cells were lysed in 1 N NaOH for 20 min at 37°C. Nonspecific uptake was assessed in the presence of 10 μmol/l cytochalasin B and subtracted from all of the measured values. The radioactivity was counted by liquid scintillation spectroscopy using a Beckman LS 5000 TD instrument (Beckman Coulter, Fullerton, CA) and normalized to protein amount measured with a Micro BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

1.3. Isolated working mouse hearts

Hearts were perfused in the isolated working heart mode with Krebs Henseleit Buffer (118.5 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 2.5 mmol/L $CaCl_2$, 0.5 mmol/L EDTA, and 5 mmol/L glucose, gassed with 95% O_2 and 5% CO_2 and supplemented with 0.4 mmol/L palmitate bound to 3% BSA) using a perfusion apparatus totally sealed to prevent loss of CO_2. Throughout the 60-min perfusion, measurements of flow and pressure (Millar pressure catheter, Millar Instruments, Houston, TX) were obtained every 20-min. Using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL), the oxygen content of freshly oxygenated buffer (arterial partial pressure of oxygen [PaO_2]) and oxygen concentration in pulmonary artery effluent, collected using a capillary tube (venous partial pressure of oxygen [PvO_2]) was measured. Palmitate oxidation was measured in one set of hearts and glycolytic flux and glucose oxidation rates were measured simultaneously in a second set of hearts. Glucose oxidation was assessed by measuring $^{14}CO_2$ released by the metabolism of [U-^{14}C] glucose (specific activity = 296 Mbq/mol). The amount of ^3H_2O released from the metabolism of exogenous [5-^{3}H] glucose (specific activity = 177 Mbq/mol) was used to determine glycolytic flux. Palmitate oxidation rates were measured by determining the amount of ^3H_2O released from [9,10-^{3}H] palmitate (specific activity = 42 Gbq/mol). The following formulas were used to determine myocardial oxygen consumption and cardiac hydraulic work: MVO_2 [ml * min-1 * g-1 WHW] = [(PaO_2-PvO_2)/100) * (Coronary flow/WHW) * (725/760) * (1000 * C)]; where PaO_2 = arterial partial pressure of oxygen [mmHg], PvO_2 = venous partial pressure of oxygen [mmHg], WHW = wet heart weight [g], 725 and 760 are atmospheric pressures at the University of Utah and at sea level respectively [mmHg], and C = Bunsen Coefficient for

plasma i.e. 0.0212. Cardiac hydraulic work [J * min-1 * g-1 WHW] = CO * DevP * 1.33 * 10^{-4} / g WHW; where CO = Cardiac output [ml/min], and DevP = Developed pressure [mm Hg] [2].

1.4. Mitochondrial Function Measurements

Left ventricular muscle fibers were dissected from freshly excised hearts and permeabilized with saponin. Respiration and ATP synthesis were measured using palmitoyl-carnitine (20μM, PC) combined with malate (2mM), and succinate (5mM) in the presence of rotenone (10mM) as substrates.

Saponin-permeabilized cardiac fibers: Mitochondrial function was measured in saponin-permeabilized cardiac muscle fibers [3]. Small pieces (2 to 5 mg) of left ventricular cardiac muscle were dissected from freshly excised hearts and permeabilized for 30 minutes at 4 °C in buffer A (50 μg/mL saponin, 7.23 mmol/L K_2EGTA, 2.77 mmol/L K_2CaEGTA, 6.56 mmol/L $MgCl_2$, 20 mmol/L imidazole, 0.5 mmol/L dithiothreitol, 53.3 mmol/L K-methanS, 20 mmol/L taurine, 5.3 mmol/L Na_2ATP, 15 mmol/L PCr, and 3 mmol/L KH_2PO_4, pH 7.1 adjusted at 25 °C). Next, fibers were washed twice for 10 minutes at 4 °C in buffer B (7.23 mmol/L K_2EGTA, 2.77 mmol/L K_2CaEGTA, 1.38 mmol/L $MgCl_2$, 20 mmol/L imidazole, 0.5 mmol/L dithiothreitol, 100 mmol/L K-methanS, 20 mmol/L taurine, 3 mmol/L KH_2PO_4, 2 mg/mL BSA, 2 mmol/L malate) supplemented with 20 μmol/L Palmitoyl-carnitine or 5 mmol/L succinate and 10 mmol/L rotenone, pH 7.1 adjusted at 25 °C).

Mitochondrial oxygen consumption: The respiratory rates of cardiac fibers were measured using an oxygen sensor probe (Ocean Optics, Dunedin, FL) in 1 ml of KCl buffer at 25 °C (125 mmol/L KCl, 20 mmol/L HEPES, 3 mmol/L Mg-Acetate, 0.4 mmol/L EGTA, 2 mg/ml BSA, 5 mmol/L KH_2PO_4 and 0.3 mmol/L Dithiothreitol, 2 mmol/L malate), and 20 μmol/L Palmitoyl-carnitine or 5 mmol/L succinate and 10 mmol/L rotenone, (pH 7.1 adjusted at 25 °C). Oxygen consumption was determined under three different conditions: in the presence of substrate alone (V_0), following ADP-stimulation (1mM; V_{ADP}) and after addition of the ATP synthase inhibitor oligomycin (1μg/ml; V_{Oligo}). The solubility of oxygen in KCl buffer was 246.87 nmol of O_2 / mL. Oxygen consumption rates were expressed as nmol of O_2 * min-1 * mg dry fiber weight^{-1}.

Mitochondrial ATP production: For measurement of ATP production, ADP was added to 1 ml of buffer B to a final concentration of 1 mmol/L. Next, 10 µl Buffer B from the respiration chamber were added to 190 µl DMSO every 10 seconds for a 1-min time period. ATP production was determined by a bioluminescence assay based on the luciferin/luciferase reaction with the ATP assay kit (Promega Corporation, Madison, WI). ATP/O ratio was determined by the ratio of ATP synthesis rates and the maximally stimulated oxygen consumption (V_{ADP}) measured directly from permeabilized cardiac fibers isolated from the same heart.

1.5. Immunoblotting analysis

For immunoblotting analysis, ~ 50 mg of frozen tissue (heart, liver or muscle) was homogenized in 200 µl Lysis buffer (50 mmol/L Hepes, 150 mmol/L NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Sodium Pyrophosphate, 100 mmol/L Sodium Fluoride and 100 µmol/L Sodium Vanadate, 1 mmol/L PMSF, 10 µg/ml Aprotinin, and 10 µg/ml Leupeptin). For immunoblotting in isolated cardiomyocytes, cells were isolated as described above (methods 1.2), and pre-plated for 30 min for adhesion of non-cardiomyocyte cells. The supernatant containing cardiomyocytes was centrifuged at 600 rpm for 1 min, and the pellet was resuspended in lysis buffer, homogenized, and centrifuged for 10 min at 1000g. For separation of cytoplasmic and plasma membrane fractions, the resultant supernatant was further centrifuged for 1 hour at 100,000g in the ultra-centrifuge. The resultant pellet containing the membrane fraction was resuspended in 50 µl of lysis buffer and the supernatant containing the cytoplasmic fraction was removed and frozen for future analysis.

1.*6. Citrate synthase activity*

Citrate synthase activity was determined spectrophotometrically using whole heart homogenates as described [4].

1.7. RNA extraction and quantitative RT-PCR

Total RNA was extracted from hearts with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) and glycogen (Qiagen Inc., Valencia, CA). RNA concentration was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (NanoDrop 1000, NanoDrop products, Wilmington, DE). Total RNA (~ 3 μg) was reverse transcribed (SuperScriptTM III Reverse Transcriptase Kit, Invitrogen, Carlsbad, CA). The resulting cDNA, Platinum Taq DNA polymerase (Invitrogen), primers, and SYBR-green (Invitrogen) fluorescent dye were transferred to a 384-well plate in triplicate and real-time polymerase chain reaction was performed with an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA). The following cycle profile was used: 1 cycle at 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 59 °C for 15 sec, 72 °C for 30 sec, and 78 °C for 10 sec, 1 cycle of 95 °C for 15 sec, 1 cycle of 60 °C for 15 sec, and 1 cycle of 95 °C for 15 sec. Data were normalized to CPHND and results are shown as fold change vs. sham operated control mice. The following primer sequences were used (information is provided in the following order: gene name, gene sequence of forward and reverse primers (5'→3') and gene bank accession number).

Actin, alpha 1, skeletal muscle (ACTA1)

CCTGTATGCCAACAACGTCA

CTCGTCGTACTCCTGCTTGG

XM_134551

Natriuretic peptide precursor type A (NPPA)

ATGGGCTCCTTCTCCATCA

CCTGCTTCCTCAGTCTGCTC

K02781

Natriuretic peptide precursor type B (NPPB)

GGATCTCCTGAAGGTGCTGT

TTCTTTTGTGAGGCCTTGGT

D16497

Solute carrier family 2 (facilitated glucose transporter), member 1 (GLUT1)

GTCCTGCTCGTATTGCTGTG

GCCTTTGGTCTCAGGGACTT

NM_011400

Solute carrier family 2 (facilitated glucose transport), member 4 (GLUT4)

GGCATGGGTTCCAGTATGT

GAGGAAAGGAGGGAGTCTGG

NM_009204

Solute carrier family 2 (facilitated glucose transporter), member 8 (GLUT8)

TTCATGGCCTTTCTAGTGACC

GAGTCCTGCCTTTAGTCTCAG

NM_019488

Solute carrier family 2 (facilitated glucose transporter), member 12 (GLUT12)

GGGTGTCAACCTTCTCATCTC

CCAAAGAGCATCCCTTAGTCTC

NM_145176

Peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α)

GTAAATCTGCGGGATGATGG

AGCAGGGTCAAAATCGTCTG

NM_008904

1.8. Malonyl-CoA measurements:

Malonyl CoA levels were measured by gas chromatography-mass spectrometry (GC-MS) at the University of Utah Metabolomics Core Facility as previously described [5].

2. REFERENCES:

[1] Belke DD, Betuing S, Tuttle MJ, Graveleau C, Young ME, Pham M, et al. Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. The Journal of clinical investigation. 2002;109:629-39.

[2] Suga H. Ventricular energetics. Physiological reviews. 1990;70:247-77.

[3] Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI, Saks VA. Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers. Biochimica et biophysica acta. 1987;892:191-6.

[4] Riehle C, Wende AR, Sena S, Pires KM, Pereira RO, Zhu Y, et al. Insulin receptor substrate signaling suppresses neonatal autophagy in the heart. The Journal of clinical investigation. 2013;123:5319-33.

[5] Minkler PE, Kerner J, Kasumov T, Parland W, Hoppel CL. Quantification of malonyl-coenzyme A in tissue specimens by high-performance liquid chromatography/mass spectrometry. Analytical biochemistry. 2006;352:24-32.

3. SUPPLEMENTAL FIGURE LEGENDS:

Online Figure 1: mRNA expression of glucose transporter genes. mRNA expression of glucose transporters GLUT1, GLUT4, GLUT8 and GLUT12 in control (Cont) and *G1KO* hearts 4 weeks after TAC. Cont shams were set at 1 (dashed line). Data are expressed as means±SEM. Significant differences were determined by ANOVA followed by Tukey multiple comparison test, using a significance level of $p<0.05$, n=5 mice/group. (*) vs. control sham (#) vs. control TAC.

Online Figure 2: **Akt activation in control and G1KO hearts following 4 weeks of TAC or sham surgery (6 hearts per group). A**- Western blot image and densitometry for Akt phosphorylation. Data are expressed as means±SEM. Significant differences were determined by ANOVA followed by Tukey multiple comparison test, using a significance level of $p<0.05$. (*) vs. sham.

Online Figure 3: **Malonyl-CoA measurements.** Malonyl-CoA levels were measured in control and G1KO mice 4 weeks after TAC. Significant differences were determined by ANOVA followed by Tukey multiple comparison test, using a significance level of $p<0.05$. (*) vs. control sham and (φ) vs. G1KO sham.

Online Figure 4: **AMPK Activation and Mitochondrial Biogenesis. A**- Western blot image and densitometry for AMPK phosphorylation **B-** mRNA expression of PGC-1α in control and G1KO hearts 4 weeks after TAC. **C-** Mitochondrial DNA quantification 4 weeks after TAC. **D-** Citrate synthase activity in heart homogenates. Data are expressed as means±SEM. Significant differences were determined by ANOVA followed by Tukey multiple comparison test, using a significance level of $p<0.05$. (*) vs. sham.

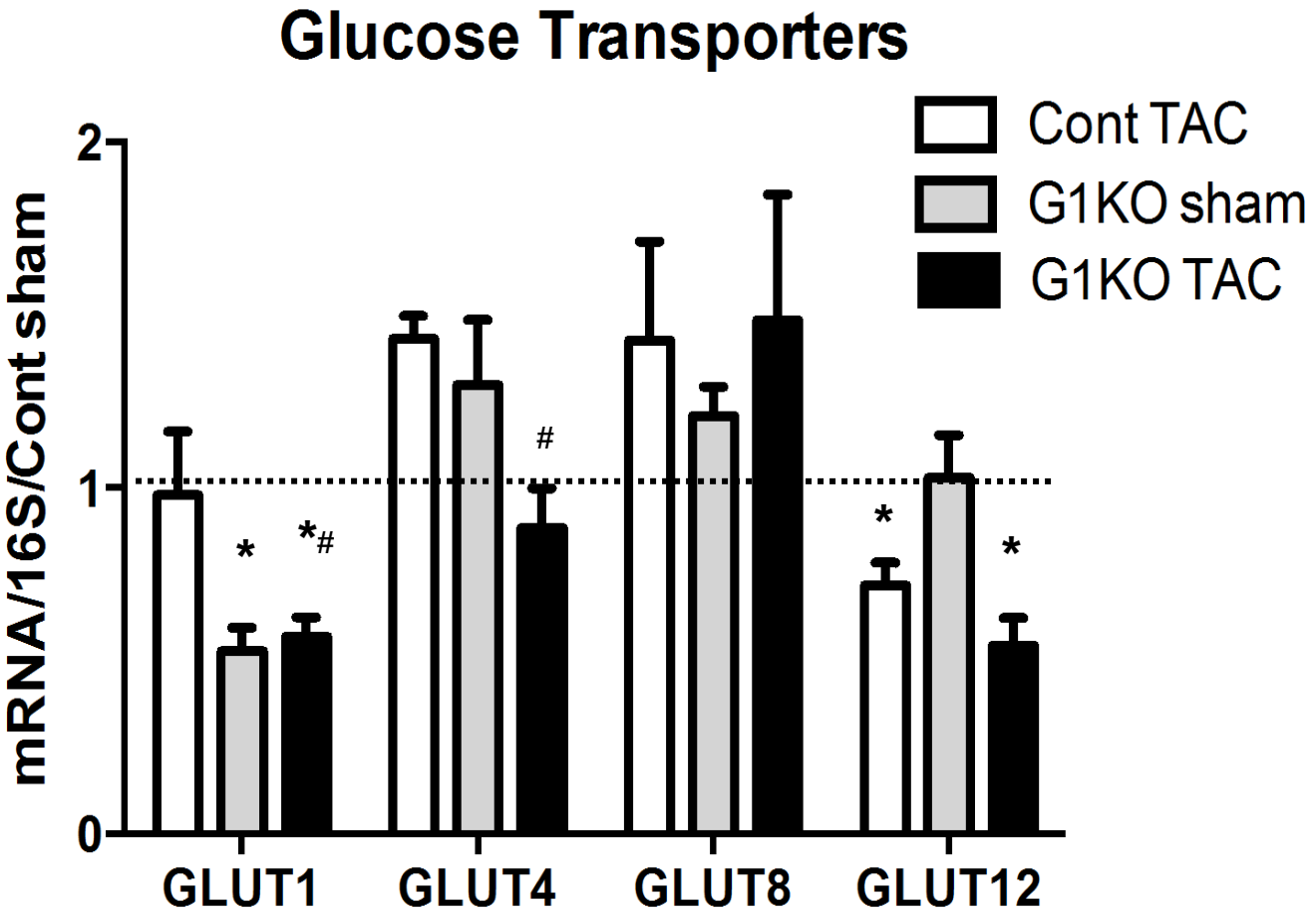
Supplemental Data Table

n≥7	Cont sham	Cont TAC	G1KO sham	G1KO TAC
Body weight (g)	26.12±0.81	24.54±1.02	27.63±0.37	24.09±0.097*
Heart weight (mg)	131.0±6.22	217.7±22.10*	130.5±5.53	224.1±30.68*
Tibia length (mm)	17.47±0.21	17.34±0.15	17.5±0.13	17.35±0.22
Wet lung weight (mg)	193±3.59	292.6±34.0*	184.3±18.1	345.5±43.1*

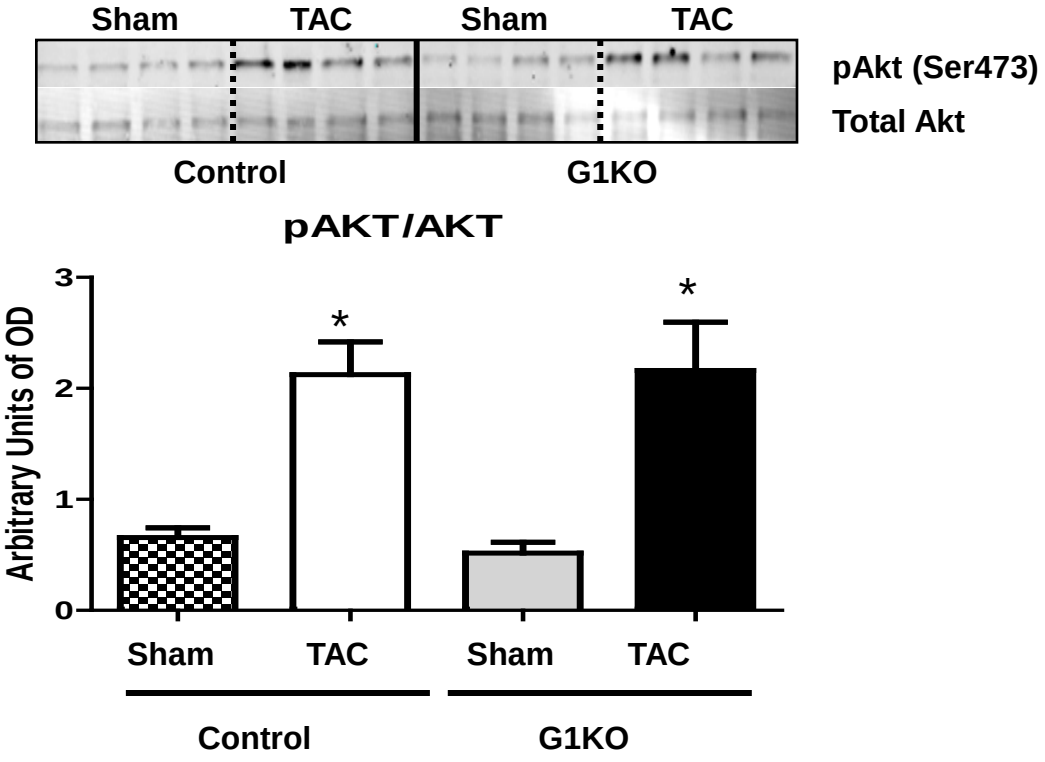
Online table 1: Mouse Characteristics 4 weeks after TAC

Significant differences were determined by ANOVA followed by Tukey multiple comparison test, using a significance level of $p<0.05$. Data are expressed as mean±SEM. (*) compared to sham.

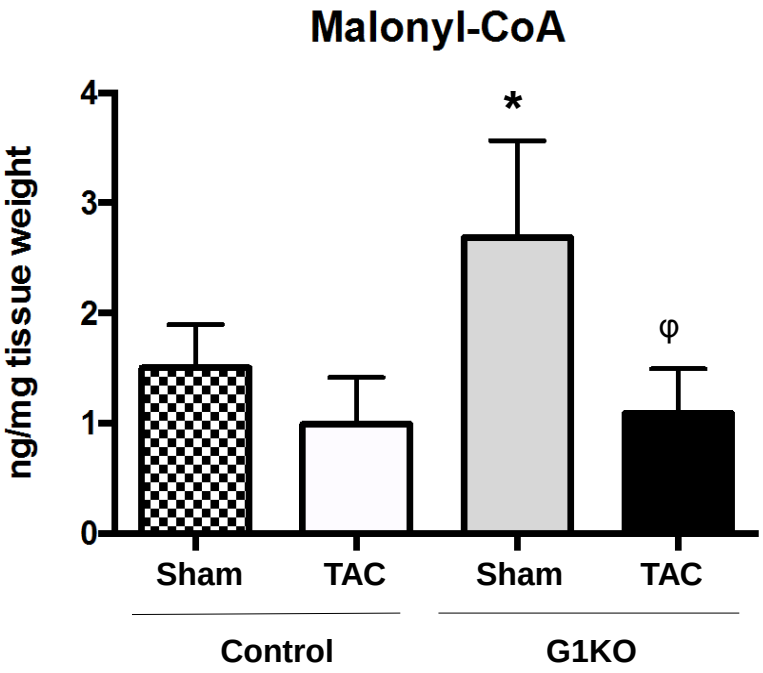
Online Figure 1



Online Figure 2



Online Figure 3



Online Figure 4