Supplemental Material

Data S1.

Supplemental Materials and Methods

Body weight and body composition analysis

Body weight were measured weekly, while food and water intakes and 24-hour urine were measure at the end of experiments. Body composition measurements were performed weekly using magnetic resonance imaging (EchoMRI-900TM, Echo Medical System, Houston, TX) to quantify lean mass, fat mass, and free water and total water content in conscious mice. The hydration ratio was calculated as Hydration% = (Total Water - Free Water) / Lean x100%.

Blood pressure measurements

We measured the systemic arterial pressure and intra-ventricular pressure using Millar catheter (SPR-839). At the end of the protocol, the mice were anesthetized by urethane (1000 mg/kg, i.p.). The systemic pressure was measured through left carotid artery and the intra-ventricular pressure was measured through the apex.

Serum ketone body measurements

The serum of mice from different groups were collected at the end of the protocol. The ketone body was measured following the manufacturer's instructions (beta Hydroxybutyrate Assay Kit, Abcam, ab83390).

Table S1. Fasting blood glucose and cardiac remodeling results.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
FBG at Week 0 (mg/dL)	91.8±11.9	97.6±5.9	91.1±6.4	90.0±3.4
FBG at Week 2 (mg/dL)	89.2±7.1	100.1±4.4	99.9±3.9	111.8±2.7
FBG at Week 4 (mg/dL)	104.6±5.0	107.3±7.3	115.1±9.2	114.6±9.1
FBG at Week 6 (mg/dL)	98.8±8.6	101.7±6.0	120.5±6.7	114.7±8.5
Heart weight/tibia length	19.36±1.03	22.19±0.51	37.55±4.68*	26.25±2.00†
Lung weight/tibia length	20.32±0.45	17.43±0.54	26.48±2.17*	21.06±0.49†
BNP (fold)	1±0.08	1.11±0.05	1.72±0.15*	1.37±0.10†
Cross sectional area in WGA staining (fold)	1±0.13	0.92±0.04	2.58±0.18*	1.54±0.09†
Masson staining	0.008±0.007	0.008±0.007	0.136±0.026*	0.029±0.012†

Results are expressed as means ± SEM. FBG: n=10-19 per group; others: n=6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures). FBG, fasting blood glucose; EMPA, empagliflozin; TAC, transverse aortic constriction; BNP, brain natriuretic peptide; WGA, wheat germ agglutinin.

Table S2. Cardiac function by echocardiography.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
Ejection fraction %	62.1±1.6	66.1±1.8	40.2±3.2*	51.3±3.8†
Fraction shortening %	33.1±1.1	35.9±1.4	18.4±1.4*	28.7±4.0†
Cardiac output ml/min	22.9±1.3	18.9±1.6	13.3±1.4*	18.9±1.9†
LVPWd	0.74±0.04	0.83±0.04	1.02±0.05*	0.85±0.05†
MPI	0.51±0.07	0.65±0.04	0.87±0.08*	0.61±0.03†
E/A ratio	1.62±0.15	1.85±0.36	4.51±1.24*	1.80±0.33†
GCS%	-23.36±1.51	-25.73±1.53	-11.17±1.12*	-18.17±2.07†

Results are expressed as means ± SEM. n=5-6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures). EMPA, empagliflozin; TAC, transverse aortic constriction; LVPWd, the end-diastolic left ventricular posterior wall thickness; MPI, myocardial performance index; E/A, E peak / A peak; GCS, global circumferential strain.

Table S3. Mice exercise capacity results.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
Running time (min)	21.3±0.9	20.4±0.9	10.8±1.3*	14.7±1.2†
VO2 (ml/kg/hr)	8089.0±88.4	7514.0±136.6	5518.0±368.6*	6681.0±156.9†
Lactate: Resting (mmol/L)	1.9±0.1	1.5±0.3	2.7±0.3*	1.7±0.2†
Lactate: Exhaustion (mmol/L)	7.7±0.7	8.0±0.9	11.7±1.1*	8.4±1.0†

Results are expressed as means ± SEM. n=5-6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures) and two-way ANOVA (repeated measures). EMPA, empagliflozin; TAC, transverse aortic constriction; VO2, oxygen consumption.

Table S4. Results of isolated cardiomyocytes.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
Resting Sarc. length (μm)	1.815±0.007	1.767±0.017	1.757±0.008	1.746±0.007
Peak ΔLengh (μm)	0.169±0.008	0.174±0.008	0.102±0.011*	0.137±0.008†
Peak shortening %	9.32±0.42	9.81±0.41	5.80±0.64*	7.86±0.46†
-dl/dt (um/sec)	-2.625±0.215	-2.350±0.116	-1.371±0.112*	-1.764±0.106†
+dl/dt (um/sec)	2.053±0.141	1.841±0.137	0.919±0.103*	1.260±0.083†
Baseline Calcium Signal (F340/380)	0.936±0.032	0.957±0.035	0.928±0.013	0.948±0.021
dF/dt(ΔF340/380/sec)	1.489±0.201	1.410±0.094	0.743±0.165*	1.531±0.262†
-dF/dt(ΔF340/380/sec)	-0.313±0.041	-0.266±0.024	-0.152±0.021*	-0.306±0.052†
Peak Calcium Signal	0.057±0.007	0.055±0.004	0.029±0.006*	0.062±0.009†
%Peak Calcium Signal	6.01±0.72	5.76±0.55	3.16±0.57*	6.51±0.95†

Results are expressed as means ± SEM. n=5-6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures). EMPA, empagliflozin; TAC, transverse aortic constriction; Sarc., sarcomere.

Table S5. Cardiac metabolism and function of isolated perfused hearts.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
Glucose oxidation (μmol/min/g dry)	1.41±0.19	1.77±0.20	0.53±0.05*	1.00±0.08†
Oleate oxidation (μmol/min/g dry)	0.25±0.04	0.29±0.03	0.14±0.01*	0.21±0.02†
Glucose uptake (μmol/min/g dry)	7.53±1.19	2.87±0.48	5.01±0.70*	2.43±0.37†
Glycolysis (μmol/min/g dry)	4.86±0.90	2.39±0.47	4.99±0.96*	2.09±0.73†
Cardiac output (ml/min)	12.36±0.05	12.57±0.15	8.92±0.21*	10.72±0.15†
Heart rate (beats/min)	367.6±14.9	386.4±22.8	328.8±25.9	383.4±20.8

Results are expressed as means ± SEM. n=5-6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures). EMPA, empagliflozin; TAC, transverse aortic constriction.

Table S6. Results of immunoblotting.

Parameter	Sham	Sham + EMPA	TAC	TAC + EMPA
CD36	1.11±0.07	0.97±0.17	0.48±0.06*	0.77±0.11†
PPARα	0.94±0.07	1.09±0.04	0.18±0.03*	0.59±0.07†
p-AMPK/AMPK	0.70±0.08	0.72±0.09	0.39±0.04*	1.06±0.07†
p-ACC/ACC	1.48±0.17	1.69±0.14	0.81±0.03*	1.51±0.12†
p-mTOR/mTOR	1.42±0.15	1.49±0.15	1.94±0.08*	1.18±0.09†
p-S6/S6	1.87±0.58	1.38±0.36	8.16±1.39*	2.88±0.51†

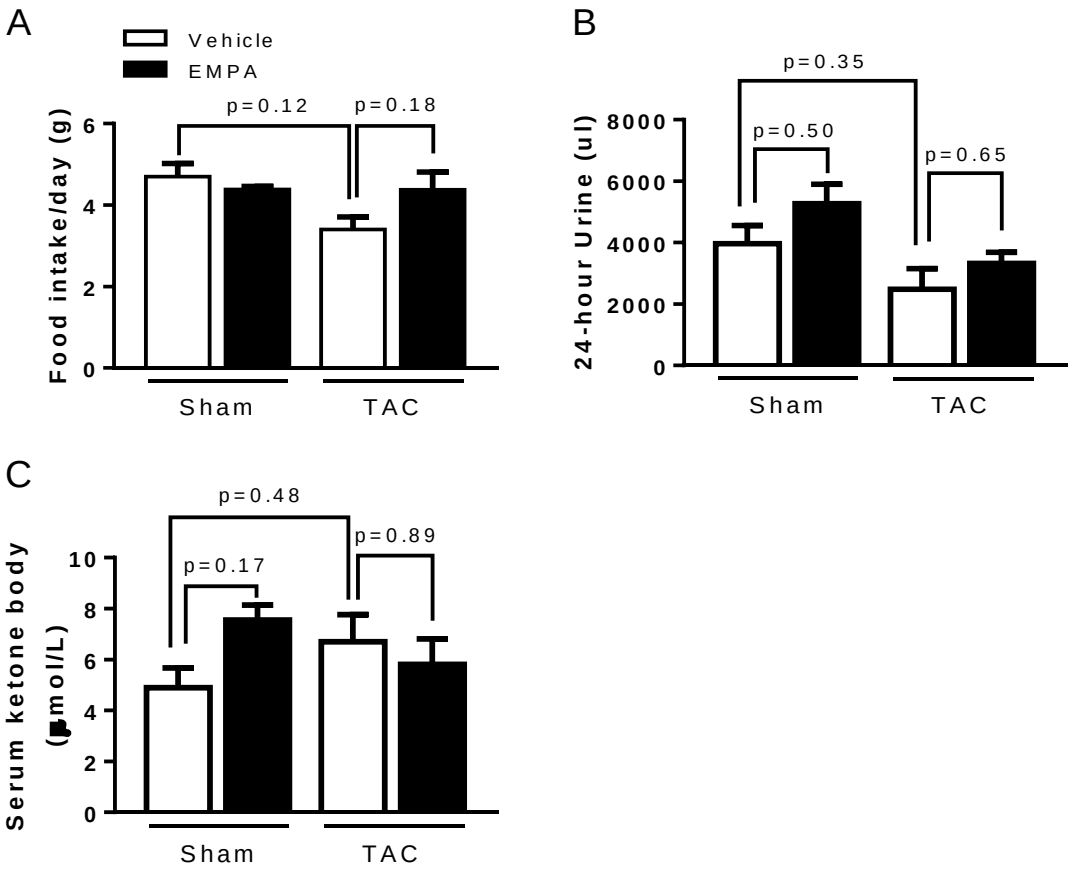
Results are expressed as means ± SEM. n=5-6 per group. *p<0.05 vs. sham group, †p<0.05 vs. TAC group. One-way ANOVA (non-repeated measures). EMPA, empagliflozin; TAC, transverse aortic constriction; CD36, cluster of differentiation 36; PPARα, Peroxisome proliferator-activated receptor α; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; mTOR, mammalian target of rapamycin.

Table S7. Acute treatment of EMPA in isolated hearts.

Parameter	Vehicle	EMPA
Mean glucose uptake (μmol/min/g dry)	5.36±0.31	3.76±0.40*
Mean glycolysis (μmol/min/g dry)	4.61±0.22	3.01±0.37*
Mean RPP (mmHg/min)	27831.0±814.7	28289.0±2589.0

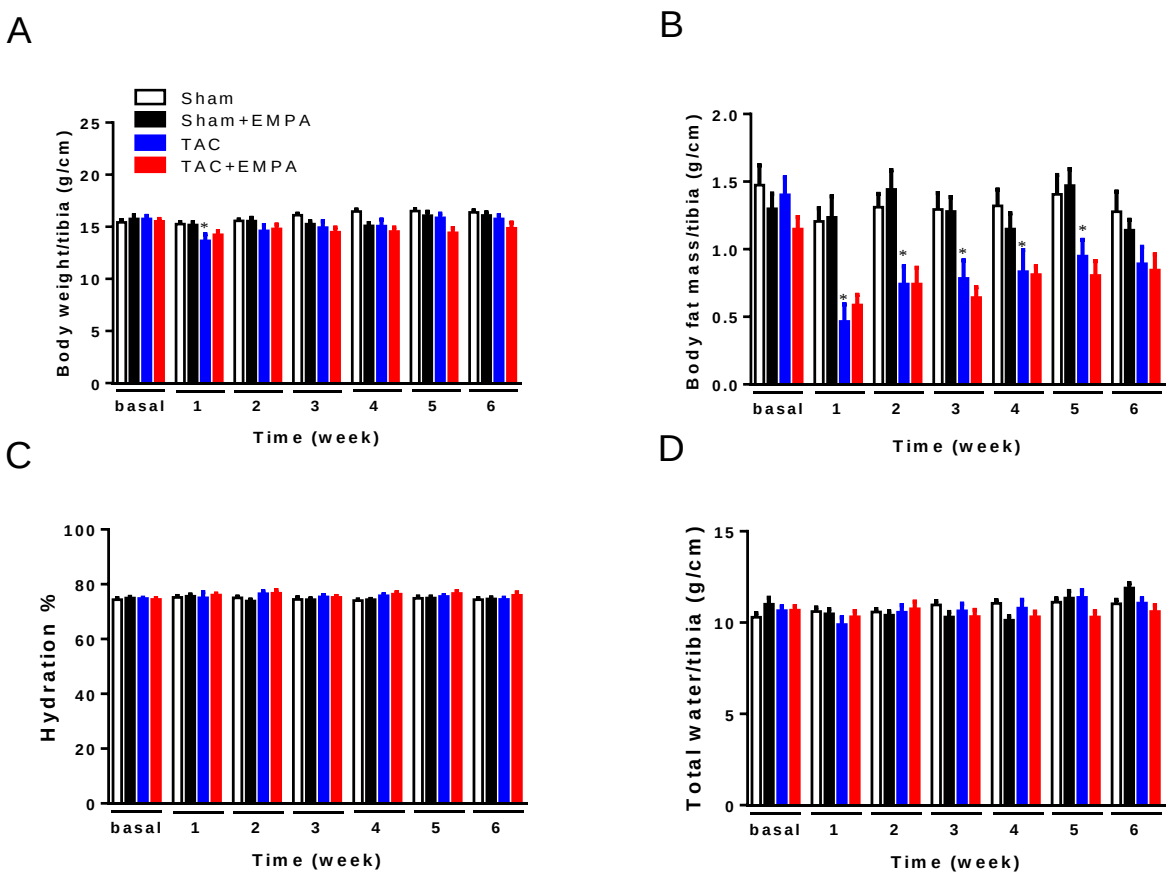
Results are expressed as means ± SEM. n=6 per group. *p<0.05 vs. Vehicle. Mann-Whitney test. EMPA, empagliflozin; TAC, transverse aortic constriction; RPP, rate pressure product.

Figure S1. Chronic EMPA treatment for 4 weeks did not significantly alter daily food intake or 24-hour urine volume.



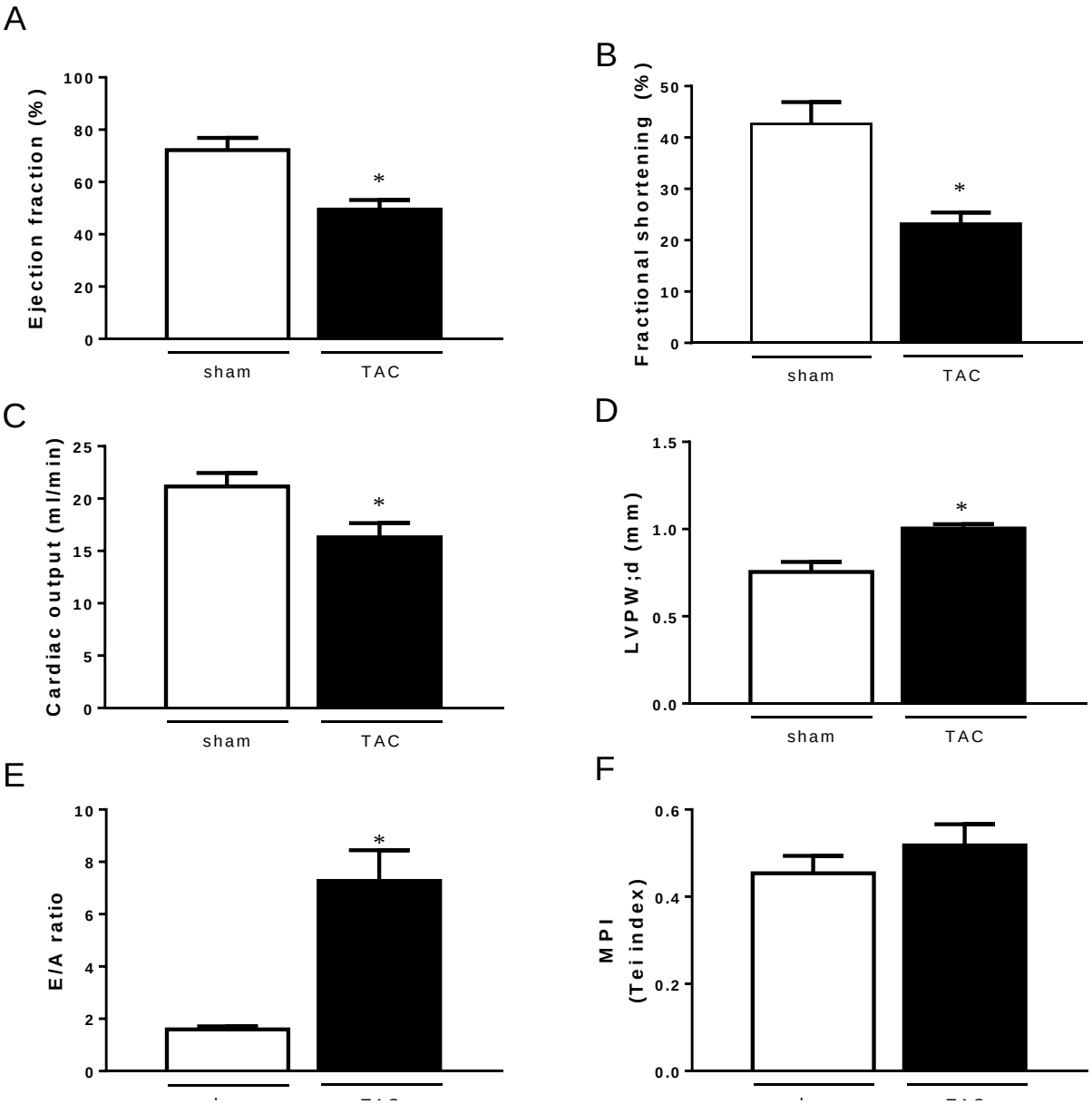
The EMPA or placebo was given to mice 2 weeks after sham or TAC surgeries. After giving EMPA for 4 weeks, we measured the average daily food intake **(A)** and 24-hour urine volume **(B)** for these mice. N=4-8 in each group. **(C)** Serum ketone body concentrations in different groups. N=7 mice per group. Results are expressed as mean ± SEM. Comparisons among groups were performed using Dunn's multiple comparison test. EMPA, empagliflozin; TAC, transverse aortic constriction; SEM, standard error of the mean.

Figure S2. Empagliflozin treatment did not significantly influence normalized body weight, fat, hydration and total water in sham or TAC groups.



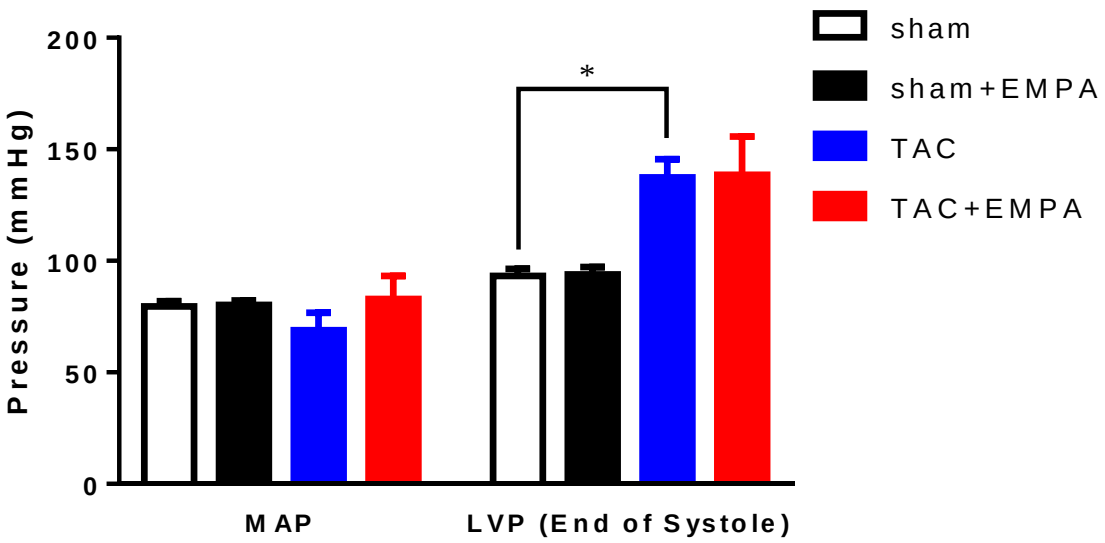
(A) Body weight normalized by tibia length over 6 weeks; **(B)** body fat normalized by tibia length; **(C)** percentage of hydration; **(D)** total body water normalized by tibia length. Results are expressed as mean ± SEM. n=10-15 in each group. *p<0.05 vs. corresponding sham group. Two-way ANOVA (non-repeated measures) (**A-D**). EMPA, empagliflozin; TAC, transverse aortic constriction; SEM, standard error of the mean.

Figure S3. Two weeks of TAC induced cardiac hypertrophy, and systolic and diastolic dysfunction.



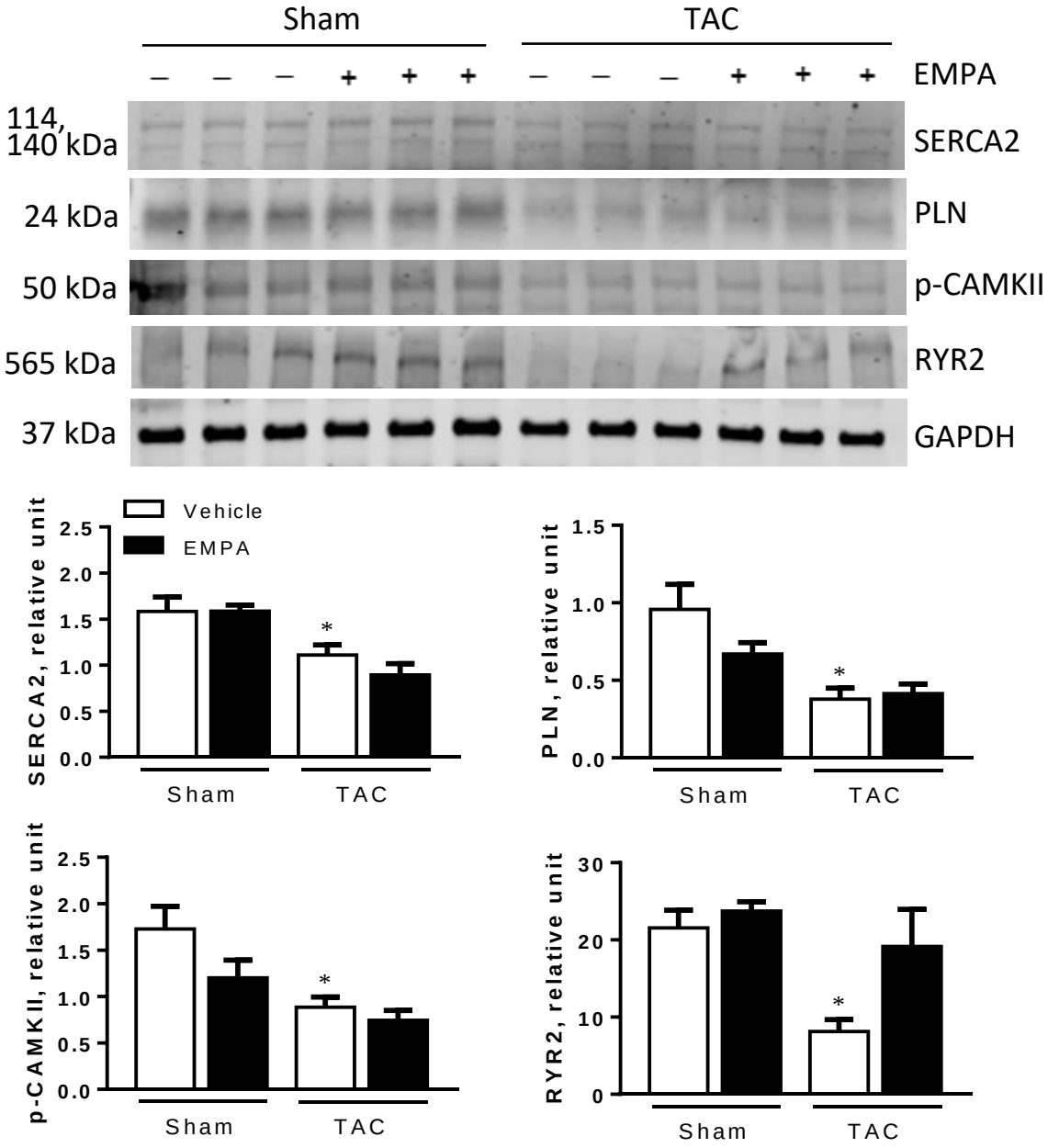
(A) ejection fraction, **(B)** fraction shortening, **(C)** cardiac output, **(D)** LVPWd, **(E)** E/A ratio, and **(F)** MPI. Results are expressed as mean ± SEM. n=10 in each group. Comparisons between groups were performed using unpaired Student *t* test. *p<0.05 vs. sham group. TAC, transverse aortic constriction; LVPWd, the end-diastolic left ventricular posterior wall thickness. MPI, myocardial performance index. SEM, standard error of the mean.

Figure S4. Empagliflozin treatment did not significantly alter mean systemic arterial pressure or left ventricular pressure (LVP) in sham or TAC groups.



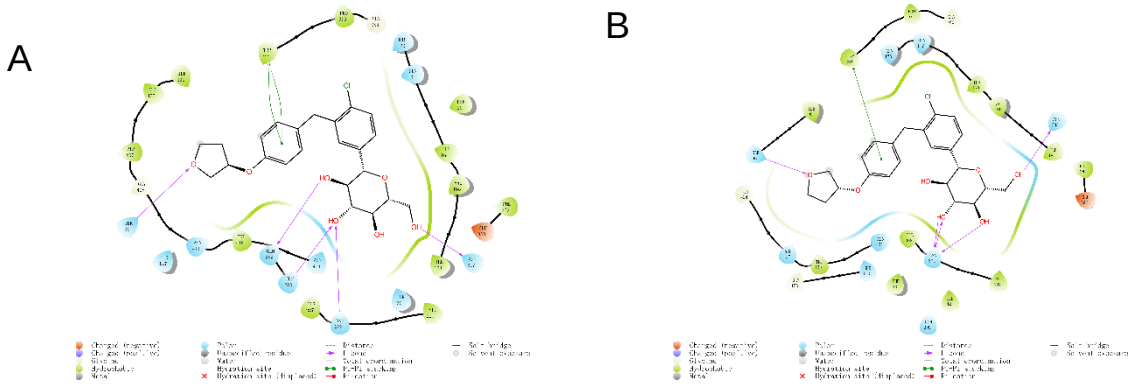
The mean arterial pressure and intra-LVP at the end of systole were measured at the end of the 4-week treatment protocol. Comparisons among groups were performed using one-way ANOVA test (repeated measures). Results are expressed as mean ± SEM. n=5 mice per group, *p<0.05 vs. corresponding sham group. MAP, mean arterial pressure; LVP, left ventricular pressure.

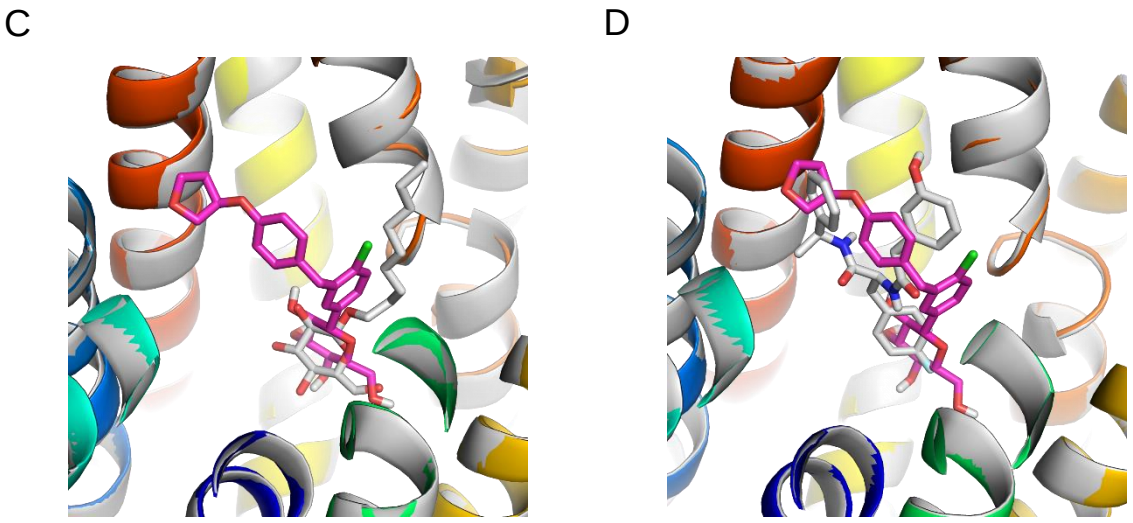
Figure S5. Immunoblotting results of calcium-related pathways.



Upper: representative blots of each protein. Lower: results of statistical analysis. Results are expressed as mean ± SEM, n=6 mice per group, *p<0.05 vs. sham, †p<0.05 vs. TAC. One-way ANOVA (non-repeated measures). SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; PLN, phospholamban; CAMKII, Calcium/calmodulin-dependent protein kinase II; RYR2, Ryanodine receptor 2; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

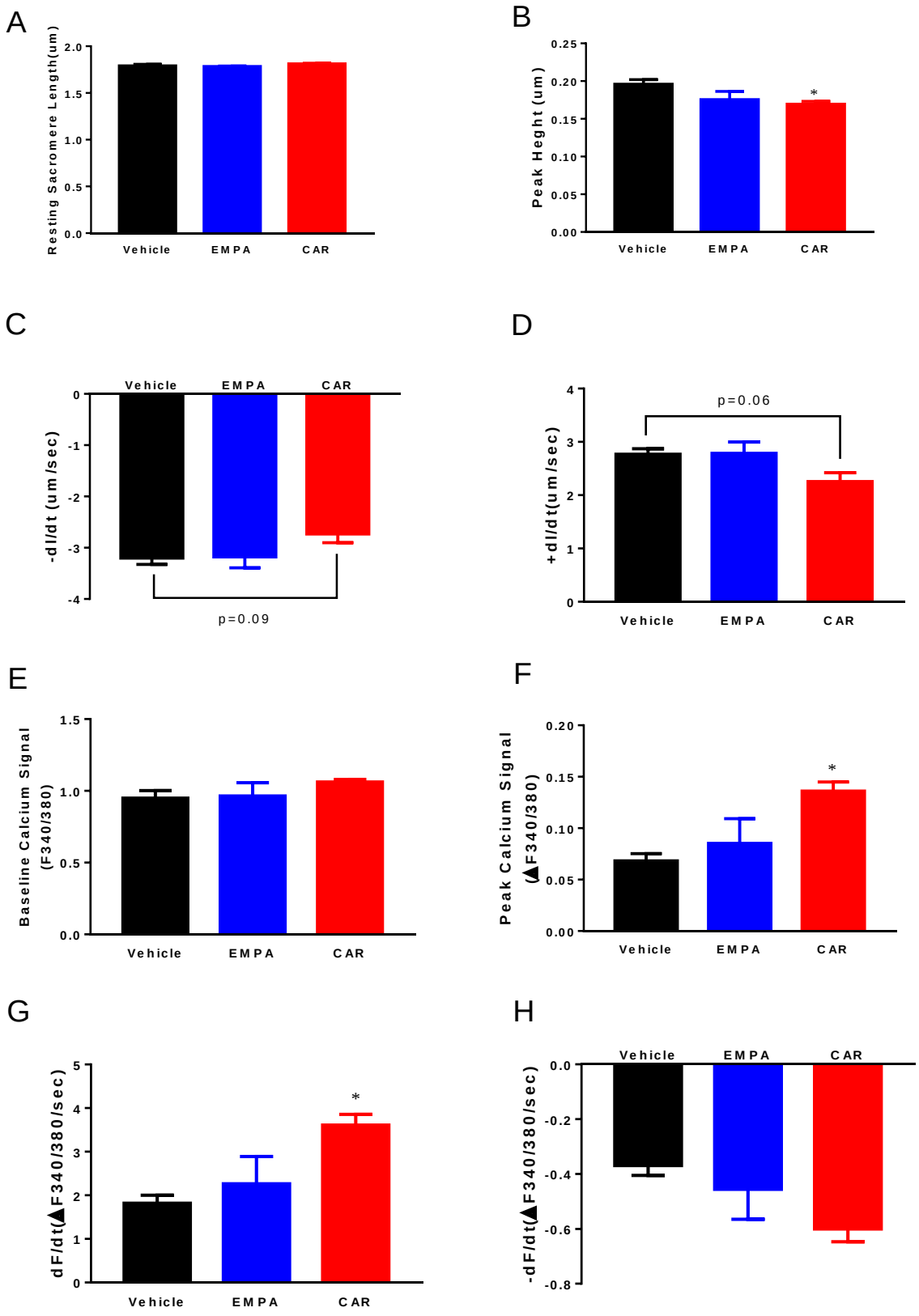
Figure S6. Additional results of molecular docking analysis.





(A) Empagliflozin forms hydrogen bonds with ASN317, GLN282, GLN288, GLN283 and SER80, and forms π-π interaction with TRP388 in GLUT1. **(B)** Empagliflozin forms hydrogen bonds with ASN333, ASN304 and SER96, and forms π-π interaction with TRP404. After compared the structure models to crystal structures. We found the binding mode of empagliflozin had highly similarity with compounds in crystal structure (PDB: 4PYP) **(C)** and (PDB: 5EQG) **(D)**. The glucoside structures in the model and crystal are almost overlapped. Asparagine; GLN, Glutamine; GLUT, glucose transporter; SER, serine.

Figure S7. Acute treatment of EMPA did not alter contractility and calcium homeostasis in isolated cardiomyocytes.



Isolated cardiomyocytes from healthy mice were treated with vehicle, EMPA (0.5μM) or CAR (0.5 μM) for 30 min. The contractility was recorded as **(A)** The resting sarcomere length; **(B)** The maximum length changed during action (Peak ∆length). **(C)** The maximum velocity of contraction (-dL/dt); **(D)** The maximum velocity of relaxation (+dL/dt); The calcium transient was recorded as **(E)** baseline calcium signal, **(F)** Peak calcium signal, **(G)** maximum velocity of calcium change during contraction (+dF/dt), and **(H)** maximum velocity of calcium change during relaxation(-dF/dt). Results are expressed as mean ± SEM, 30 cells/mouse, n=3 in each group. Comparisons among groups were performed using Dunn's multiple comparison test. EMPA, empagliflozin; CAR, cariporide; SEM, standard error of the mean.