ESM methods

Transverse aortic constriction

Heart failure was induced in 12±2 week-old mice by transverse aortic constriction. Mice were anesthetized using intraperitoneal injections of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl 0.05 mg/kg body weight). A horizontal incision (1-1.5 cm) at the suprasternal notch was used to display the transversal aorta. A 27 gauge needle was tied against the aorta using a 6-0 non-absorbable suture. After removal of the 27 gauge needle, the skin was closed and the mice were kept on a heating plate (37° C) until recovery from anesthesia. At the end of the surgery, anesthesia was antagonized using intraperitoneal injections of atipamezol (2.5 mg/kg), flumazenil (0.5 mg/kg) and buprenorphine (0.1 mg/kg body weight). For analgesia, metamizole (1.33 mg/ml) was added to the drinking water 2 days before surgery and supplied for 7 days after operation. In addition, buprenorphine (60 µg/kg body weight) was administered s.c. 1 hour before surgery. Animals that died during surgery or within 48 h were excluded from the study (not reported; on average 15% of all operated mice for TAC). In case of disturbed wound-healing, animals were killed at the discretion of the veterinarian and excluded from the study. At the end of the experiments mice were killed under isoflurane anaesthesia (5%) by cervical dislocation.

Echocardiography

Transthoracic echocardiography was performed by blinded investigators using a Vevo3100 (VisualSonics, Toronto, Ontario, Canada) system with a 30 MHz center frequency transducer as described previously (1). The animals were anesthetized with isoflurane (induction with 3%, maintenance with 1.5%), while temperature, respiration, and ECG were continuously monitored. Two-dimensional cine loops with frame rates of >200 frames/s of a long axis view and a short axis view at mid-level of the papillary muscles as well as M-mode loops of the short axis view were recorded. Thicknesses of the septum, the anterior and posterior myocardial wall, the inner diameter of the left ventricle (LVEDD) and the area of the left ventricular cavity were measured in systole (sys) and diastole (dia) from the short axis view according to standard procedures. Maximal left ventricular length was measured from the long axis view. Systolic and diastolic left ventricular volumes were calculated using the area-length method (2) and the ejection fraction was derived.

Cell isolation and culture

Isolation of murine cardiomyocytes was performed as previously described (3,4). Briefly, mice were anaesthetized with isoflurane. After death by cervical dislocation, hearts were quickly excised, mounted on a Langendorff perfusion apparatus and retrogradely perfused with nominally Ca^{2+}-free solution containing (in mmol/l) NaCl 113, KCl 4.7, KH_2PO_4 0.6, $Na_2HPO_4x2H_2O$ 0.6, $MgSO_4x7H_2O$ 1.2, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, taurine 30, BDM 10, glucose 5.5, phenol-red 0.032 for 4 min at 37°C (pH 7.4). Then, 7.5 mg/ml liberase TM (Roche diagnostics, Mannheim, Germany), trypsin 0.6%, and 0.125 mmol/l $CaCl_2$ were added to the perfusion solution. Perfusion was continued for 3-4 min until the heart became flaccid.

Ventricular tissue was collected in perfusion buffer supplemented with 5% bovine calf serum, cut into small pieces, and dispersed by repeatedly pipetting until no solid cardiac tissue was left. Ca^{2+}-reintroduction was performed by stepwise increasing [Ca^{2+}] from 0.1 to 1.0 mmol/l.

Left ventricular tissue of explanted hearts from heart transplant recipients was used. Chunk isolation was performed as described previously (5) with sliced tissue incubated at 37°C in a spinner flask filled with Joklik MEM solution with collagenase 2 and trypsin (0.5%). After 45 min, the supernatant was discarded and the remaining tissue poured into the flask.
Digestion was continued with fresh JMEM solution containing collagenase 2 for 10-15 min until myocytes were disaggregated. Solutions containing disaggregated cells were centrifuged (with low g, 60 rpm, 3 min), and the pellet was resuspended in fresh JMEM solution containing collagenase 2. The suspension was poured back into the flask for further digestion (15 min). This procedure was repeated 4-5 times. Cells were then stored in solution that stops enzyme activity containing JMEM and BCS (10%) at room temperature. Only elongated cells with cross striations and without granulation were selected for experiments.
Cells were cultured as described previously (6,7) for 24 h with 1 µmol/l empagliflozin. For this, a 10 mM empagliflozin stock in DMSO was prepared. For culture, stock was diluted at 1:10000 for a final working concentration of 1 µmol/l. This results in a solvent (DMSO) concentration of 0.01% in the measurement buffer, which is 10 times smaller as the maximal acceptable concentration of DMSO usually used in the literature (8). For vehicle buffers, we added exactly the same amount of DMSO (i.e. 0.01%).

Before and after culture, two different methods to test cell viability were employed. Live cardiomyocytes were counted using a Neubauer counting chamber and were additionally imaged in prespecified locations on the cell culture chambers. The comparison of vehicle control and empagliflozin cultures did not show significant differences in the percentage of viable cardiomyocytes (supplemental figure 1). Fasentin-treatment (50 µmol/L) did also not affect cardiomyocyte survival.
Measurements were performed after 24 h exposure to empagliflozin (1 µmol/l) or vehicle control.

Western blot analysis

Cell lysates were used. After denaturation for 30 min at 37°C in 1% β-mercaptoethanol, proteins were separated on 8% SDS-polyacrylamide gels, then transferred to a nitrocellulose membrane (or PDVF membrane) and incubated with primary antibodies: rabbit monoclonal anti-GLUT1 (1:25000, ab115720, Abcam, Cambridge, UK), rabbit monoclonal anti-GLUT4 (1:1000, Abcam ab188317, Abcam, Cambridge, UK), and mouse monoclonal anti-GAPDH (1:10000, g8795, Sigma Aldrich, St. Louis, Missouri, USA) at 4˚C overnight. Buffers for the antibodies consisted of Tris-buffered saline with Tween20 (TBST) and 5% milk powder. TBST was prepared with 2 mmol/l Tris, 15 mmol/l NaCl. Tween20 was then added 1:1000.

Secondary antibodies were HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgG (anti-rabbit 1:5000, NA934; anti-mouse 1:10000; NA931; GE Healthcare, Little Chalfont, UK) that were incubated for 1 h at room temperature. For

chemiluminescent detection, Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Darmstadt, Germany) was used.

Glucose concentration assay

Cardiomyocyte glucose concentration was evaluated using the Abcam Glucose Assay Kit (ab65333, Abcam, Cambridge, UK). Cardiomyocytes were isolated and cultured as described above. Cells were settled for 20 min and supernatant was discarded. Cells were then washed with phosphate-buffered saline (PBS) and resuspended in the provided assay buffer. After centrifugation a low g for 2 min at 4°C, supernatant was transferred to new tubes and kept on ice. Perchloric acid/potassium hydroxide deproteinization was performed as described by the manufacturer. For the assay, a glucose standard was prepared and all materials equlibrated to room temperature. Samples were added to a 96 well plate and volume adjusted using the provided glucose assay buffer. 50 µl reaction buffer was added to each well (background reaction mix to control wells). The plate was incubated for 30 min at 37°C and then fluorescence was analyzed using a Tecan plate reader at 535 nm excitation/ 587 nm emission.
Background from negative controls (without reaction mix) was substracted from the measurements (however, background was always less than 1% of test sample fluorescence). A standard curve was plotted from the glucose standard and [glucose] in the samples calculated according to this curve. A mean cellular volume of 30 pl per cardiomyocyte was assumed (9) for calculations of glucose concentrations relative to cellular volume.

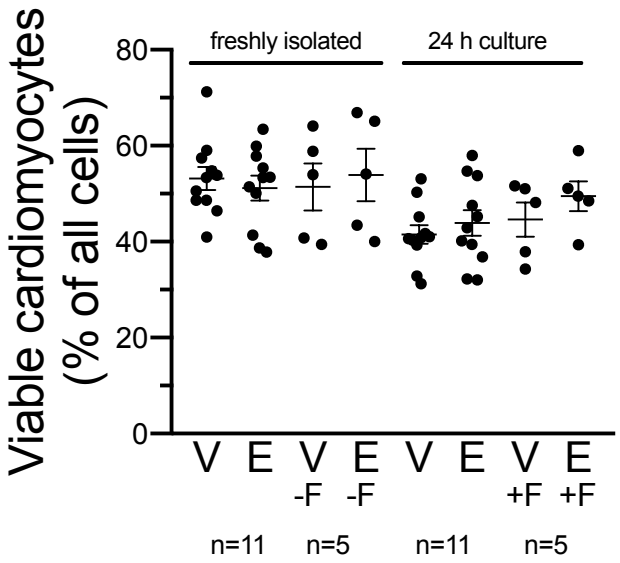
Glucose uptake assay

Cardiomyocyte glucose uptake was evaluated using the Abcam Glucose Uptake Assay Kit (ab136956, Abcam, Cambridge, UK). Cardiomyocytes were isolated and cultured as described above. After culture, cells were centrifuged at low g for 3 min and supernatant was discarded.
Cells were washed twice with PBS (containing 1 mmol/l Ca^{2+}) and centrifuged. Supernatant was discarded and cells were exposed to normal Tyrode's solution with 10 mmol/l mannitol (instead of glucose) for 40 min to starve them of glucose. 2-Deoxyglucose (10 mmol/l) was added and in some cases fasentin 50 µmol/l (F5557, Sigma Aldrich, St. Louis, Missouri, USA) was added to specifically inhibit GLUT1 (experimental groups: vehicle, empagliflozin, vehicle + fasentin, empagliflozin + fasentin) for 20 min. 2-Deoxyglucose was omitted in respective negative controls. Cells were again centrifuged at low g for 3 min and the supernatant was discarded. Cells were washed with PBS and supernatant was discarded. Cells were then lysed with the supplied extraction buffer and frozen in liquid nitrogen. The rest of the protocol was performed as detailed in the manufacturer's protocol. In brief, cell lysates were thawed and then heated at 85°C for 40 min. Cells were then cooled on ice for 5 min and neutralized with 10 µl neutralization buffer. After spinning for 2 min at 500 rpm, the supernatant was transferred to new tubes. Samples were then added to a 96 black well plate and volume was adjusted with 2DG-Uptake-Assay-Buffer. After addition of reaction mix, plates were incubated in the dark at 37°C for 40 min. Then fluorescence was measured at 535 nm excitation/ 587 nm emission using a Tecan plate reader.

Zero standard and background from negative controls (cells not treated with 2-Deoxyglucose) was substracted from the measurements (however, background was always less than 1% of test sample fluorescence). A standard curve was plotted from the 2-Deoxyglucose-6-phosphate standard and uptake in the samples calculated according to this curve. A mean cellular volume of 30 pl per cardiomyocyte was assumed (9) for calculations of 2-Deoxyglucose uptake relative to cellular volume.

ESM figures

ESM figure 1: cell survival



ESM figure 1 legend:

Percentage of viable cardiomyocytes immediately after cell isolation and after 24 h cell culture. Cells were imaged in three prespecified areas per cultured chamber and viable elongated cross-striated cardiomyocytes were counted against non-viable round-shaped cells without cross striation. The mean percentage for each isolation is shown. V=vehicle, E=empagliflozin, -F=destined for fasentin-treatment but not yet exposed, +F=exposed to fasentin (50 µmol/l). Cell survival was not different between vehicle or empagliflozin and unaffected by fasentin-treatment (p=n.s.; Kruskal-Wallis-test with Dunn's multiple comparisons post-test).

ESM tables

ESM table 1: Human data

Ventricular failing cardiomyocytes:

Nr	Age	Sex	BMI	EF	LVEDD	Rhythm	Previous surgery	Valvular dysfunction	Diabetes/ HbA1c %	CAD	Art Hyp	KD	Creatinine	GFR	NTpro BNP
1	22	m	22.1	20	49	SR	none	None	n	n	y	1	1.38	72	11341
2	67	m	31.3	14	70	SR	none	MR I°, TR I°	y/6.4	y	y	1	2.21	30	4017
3	59	m	25.5	13	68	SR	none	MR III°, TR II°	n	n	y	1	1.69	43	14872
4	43	m	23.4	30	60	SR	none	TR I°	n	n	y	1	1.38	62	1189

Table 1 legend: Clinical characteristics of individuals, whose left ventricular tissue was used (explanted hearts from individuals with end-stage heart failure undergoing transplantation). **y**=yes, **n**=no**, Nr**=number, **CAD**=coronary artery disease, **EF**= ejection fraction in %, **LVEDD**=left ventricular enddiastolic diameter in mm, m=male, **SR**=sinus rhythm, **CABG**=coronary artery bypass graft, valves=valvular dysfunction, **MR**=mitral regurgitation, **TR**=tricuspid regurgitation **x°**=indicated severity degree according to international standards, **Art Hyp**=arterial hypertension, **KD**=kidney disease, **GFR**=glomerular filtration rate in ml/min, **NTproBNP** in pg/ml (internal cutoff <150 pg/ml)

ESM table 2: TAC mice:

	Pre-OP	5 w post-TAC
Heart rate (bpm)	443.6 ± 9.1	469.7 ± 21.5
Ejection fraction (%)	59.8 ± 3.9	31.4 ± 4.5*
Fractional shortening (%)	31.5 ± 2.8	14.6 ± 2.3*
Stroke volume (µl)	37.4 ± 2.7	19.8 ± 3.1*
LV AWThd (mm)	1.0 ± 0.01	1.23 ± 0.07*
LV PWThd (mm)	0.94 ± 0.03	1.39 ± 0.25
LVIDs (mm)	2.7 ± 0.1	3.3 ± 0.3
LVIDd (mm)	3.8 ± 0.1	3.8 ± 0.2

Table 2 legend: Echocardiographic data obtained from 4 mice. OP=TAC surgery, bpm=beats per minute, LV=left ventricle, AWThd= anterior wall thickness in diastole, PWThd= posterior wall thickness in diastole, LVIDs= left ventricular internal diameter in systole, LVIDd= left ventricular internal diameter in diastole, data shown as mean±SEM, *P<0.05 versus respective pre-OP-measurement (student's t-test)

Supplemental references

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