Activation of IGF-1 Receptors/Akt signaling by systemic hyperinsulinemia contributes to cardiac hypertrophy but does not regulate cardiac autophagy in obese diabetic mice

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Supplemental Material

Amino acids quantification. Frozen heart samples were digested in cold 90% methanol solution containing 1 µg of d4-succinic acid. The samples were extracted using an Omni Bead Ruptor followed by incubation at -20˚C freezer for 1 hr. After incubation centrifuge the sample tubes at 20,000 x g for 10 minutes at 4˚C. The supernatant was transferred from each sample tube into a labeled, fresh microcentrifuge tubes. Pooled quality control samples were made by removing 15% volume of collected supernatant from each sample. The samples were dried *en vacuo*.

GC-MS analysis and data analysis. All GC-MS analysis was performed with a Waters GCT Premier mass spectrometer (Beverly, MA) fitted with an Agilent 6890 gas chromatograph (Santa Clara, CA) and a Gerstel MPS2 autosampler (Linthicum Heights, MD). Dried samples were suspended in 40 µL of a 40 mg/mL O-methoxylamine hydrochloride (MP Biomedicals, Solon, OH) in pyridine (Thermo Fisher) and incubated for one hour at 30°C. To autosampler vials was added 25 uL of this solution. 40 µL of N-methyl-N-trimethylsilyltrifluoracetamide (Thermo Fisher) was added automatically via the autosampler and incubated for 60 minutes at 37°C with shaking. After incubation 3 µL of a fatty acid methyl ester standard (FAMES) solution was added via the autosampler then 1 uL of the prepared sample was injected to the gas chromatograph inlet in the split mode with the inlet temperature held at 250°C. A 10:1 split ratio was used for analysis. The gas chromatograph had an initial temperature of 95°C for one minute followed by a 40°C/min

ramp to 110°C and a hold time of 2 minutes. This was followed by a second 5°C/min ramp to 250°C, a third ramp to 350°C, then a final hold time of 3 minutes. A 30 m Phenomex ZB5-5 MSi column with a 5 m long guard column was employed for chromatographic separation. Helium was used as the carrier gas at 1 mL/min. Due to the high amounts of several metabolites the samples were analyzed once more at a 10-fold dilution. Data was collected using MassLynx 4.1 software (Waters). Metabolites were identified and their peak area was recorded using QuanLynx (Waters). This data was transferred to an Excel spread sheet (Microsoft, Redmond WA). Metabolite identity was established using a combination of an in house metabolite library developed using pure purchased standards and the commercially available NIST library.

Western-blotting. Total protein extraction was performed as previously described (1). Proteins were resolved by SDS-PAGE and electro-transferred to nitrocellulose, for high molecular weight proteins (>150kDa,) or PVDF, for low molecular weight proteins (<150kDa). The list of primary antibodies used is described in **Supplemental Table 1**. Protein detection was carried out with Alexa Fluor anti-rabbit 680 and anti-mouse 800 (LI-COR) as secondary antibodies, and fluorescence was quantified using the LI-COR Odyssey imager.

Reference

(1) Belke, D.D., Betuing, S., Tuttle, M.J., Graveleau, C., Young, M.E., Pham, M., Zhang, D., Cooksey, R.C., McClain, D.A., Litwin, S.E., et al. 2002. Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. *J Clin Invest* 109:629-639.

Supplemental Figure Legends

Supplemental Figure 1. Impaired autophagic flux in the hearts of diet-induced obese (DIO) mice. (A) Representative western-blots of microtubule associated protein 1 light chain 3 (LC3) I and LC3II, p62/SQSTM1, autophagy related 3 (ATG3) and glyceraldehyde dehydrogenase (GAPDH) in whole heart homogenates from normal chow diet (NCD) and high fat diet (HFD) fed mice. NCD and HFD mice received an intra-peritoneal injection of saline or 50 mg/kg body weight chloroquine (CQ) for 4 hours before sacrifice. **(B)**, **(C)**, **(D)** and **(E)** The corresponding densitometry of LC3II/GAPDH, LC3-II/I ratios, p62/GAPDH and ATG3/GAPDH expressed as fold-change relative to NCD. Data are means ± SEM. N=3-4 mice per group. * $P < 0.05$ versus NCD under the same treatment condition; # $P < 0.05$ versus saline within the same diet.

Supplemental Figure 2. Preserved cardiac autophagy in CIRKO mice. (A) Representative western-blots of LC3 I and LC3II, ATG3, p62/SQSTM1 and GAPDH in whole heart homogenates from wild-type and CIRKO mice. **(B)** The corresponding densitometry of LC3-II/I ratios, LC3I/GAPDH, LC3II/GAPDH, ATG3/GAPDH and p62/GAPDH expressed as fold-change relative to wild-type. Data are means ± SEM. N=3 mice per group.

Supplemental Figure 3. Levels of amino acids in hearts of wild-type, cIGF-1R+/-, (*ob/ob*) and (*ob/ob*)/IGF-1R^{+}/$^{-}$ mice. Data are means ± SEM. N=4 mice per group.

Supplemental Table 1: List of antibodies used

Antibody	*Source*	*Dilution*
Akt (pan)	Cell Signaling	1:1000
AMPKα	Cell Signaling	1:1000
ATG3	Sigma	1:1000
ATG5/12	Cell Signaling	1:1000
ATG7	Cell Signaling	1:1000
GAPDH	Cell Signaling	1:1000
IGF-I Receptor β	Cell Signaling	1:1000
LC3B	Sigma	1:1000
mTOR	Cell Signaling	1:1000
Phospho-44/42 MAPK (Erk1/2)	Cell Signaling	1:1000
P62/SQSTM1	Abcam	1:1000
Phospho-70 S6 Kinase	Cell Signaling	1:1000
P90RSK (Ser359/363)	Cell Signaling	1:1000
Phospho-Akt (Ser473)	Cell Signaling	1:1000
Phospho-Akt (Thr308)	Cell Signaling	1:1000
Phospho-AMPKα (Thr172)	Cell Signaling	1:1000
Phospho-IGF-I Receptor β (Tyr1135)	Cell Signaling	1:1000
Phospho-mTOR (Ser2448)	Cell Signaling	1:1000

Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling	1:1000
Phospho-p70 S6 Kinase (Thr389)	Cell Signaling	1:1000
Phospho-S6 (Ser235/236)	Cell Signaling	1:1000
Phospho-ULK-1 (Ser757)	Cell Signaling	1:1000
Rab 9	Cell Signaling	1:1000
RSK1/2/3	Cell Signaling	1:1000
S6	Cell Signaling	1:1000
ULK-1	Cell Signaling	1:1000

Supplemental Table 2: Ventricular parameters in wild-type and (*ob/ob*) mice treated with saline or chloroquine

	Wild-type		***(ob/ob)***	
	Saline *(n=5)*	***Chloroquine*** *(n=5)*	***Saline*** *(n=5)*	***Chloroquine*** *(n=5)*
LVPWd (mm)	0.67 ± 0.03	0.65 ± 0.03	0.71 ± 0.04	0.7 ± 0.05
LVPWs (mm)	0.9 ± 0.03	0.9 ± 0.03	1.1 ± 0.03*	1.2 ± 0.09*
LVIDd (mm)	3.8 ± 0.06	3.7 ± 0.07	3.7 ± 0.01	3.4 ± 0.1
LVIDs (mm)	2.6 ± 0.08	2.6 ± 0.07	2.5 ± 0.1	2.3 ± 0.07*#
FS (%)	32.1 ± 1.1	30.6 ± 1.3	31 ± 2	30.5 ± 2.5

Values are mean ± SEM. * $p < 0.05$ *versus* wild-type under the same treatment condition and $^{\#}$ $p < 0.05$ *versus* saline within the same genotype. LVPWd: left ventricular posterior wall in diastole; LVPWs: left ventricular posterior wall in systole; LVIDd: left ventricular interior diameter in diastole LVIDs: left ventricular interior diameter in systole; FS: fractional shortening.

Supplemental Table 3: Ventricular parameters in wild-type and (*ob/ob*) mice treated with DMSO or PD98059 (PD)

	Wild-type		***(ob/ob)***	
	DMSO *(n=5)*	***PD*** *(n=5)*	***DMSO*** *(n=5)*	***PD*** *(n=5)*
LVPWd (mm)	0.84 ± 0.05	0.87 ± 0.04	0.79 ± 0.04	0.8 ± 0.02
LVPWs (mm)	0.92 ± 0.05	1.05 ± 0.05	0.95 ± 0.03	0.92 ± 0.04
LVIDd (mm)	3.79 ± 0.05	3.7 ± 0.07	3.14 ± 0.2**	3.51 ± 0.07
LVIDs (mm)	2.97 ± 0.06	2.89 ± 0.1	2.42 ± 0.16*	2.7 ± 0.07
FS (%)	21.47 ± 1.5	21.29 ± 2.2	23.16 ± 0.52	22.93 ± 0.7
Heart Rate (bpm)	414.4 ± 17.1	437.4 ± 28.6	385.7 ± 17.3	403.9 ± 18.9

Values are mean ± SEM. * $p < 0.05$; ** $p < 0.005$ *versus* wild-type under the same treatment condition. LVPWd: left ventricular posterior wall in diastole; LVPWs: left ventricular posterior wall in systole; LVIDd: left ventricular interior diameter in diastole LVIDs: left ventricular interior diameter in systole; FS: fractional shortening.