Supplemental Material

FOXO1-mediated Upregulation of Pyruvate Dehydrogenase Kinase-4 (PDK4) Decreases Glucose Oxidation and Impairs Right Ventricular Function in Pulmonary Hypertension: *Therapeutic Benefits of Dichloroacetate*

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Methods

Echocardiography Echocardiography was performed on lightly anesthetized, spontaneously breathing rats (isoflurane 2.0%) using the Vevo 2100 System for animal models (VisualSonics Inc, Ontario, Canada). Pulmonary artery acceleration time (PAAT), RV free wall thickness (RVFW), cardiac output (CO) and stroke volume (SV) Doppler, and 2D and M-mode echo were measured as previously described[1]. For quantification of CO, maximal diameter of pulmonary artery (PAd) and velocity time integral (VTI_{PA}) of pulsed wave Doppler were obtained. CO was then calculated as: 3.14 x $(PAd/2)^2$ X VTI_{PA} x heart rate. Tricuspid annular plane systolic excursion (TAPSE) was measured as a marker of right ventricle function. TAPSE of the tricuspid annular plane was measured using M-mode through the lateral portion of the tricuspid annular plane in the apical four-chamber view[2].

Cardiac output and Catherization Cardiac output and RV systolic pressure (RVSP) in rats was measured using the thermodilution method in closed-chest, anesthetized rats, as described previously[3]. Briefly, after anesthesia and intubation, the right carotid artery and jugular vein was exposed. After the distal part of the carotid artery was ligated, a thermistor probe catheter (Physitemp Instruments, Inc., Clifton, NJ) was gently inserted into the aorta via the carotid artery and a 22-gauge catheter was gently introduced into the jugular vein via a cut-down technique. A bolus of iced saline (0.3ml) was injected and a thermodilution curve (temperature vs. time) was recorded. PowerLab data acquisition system (AD Instruments, Colorado Springs, CO) was used to record temperature change

and CO was then determined by calculating the area under the temperature curve. Subsequently the chest was opened for direct RV puncture and catheterization of the pulmonary artery (PA).

Energy Metabolism Measurements in Isolated Working Hearts Cardiac output (mL/min) was expressed in terms of m^3/s as $1mL = 1 \cdot 10^{-6}$ m^3 and 1min = 60s; developed pressure (mmHg) was expressed in Pa as 1mmHg = 133.322 Pa = 133.322 $kg/(m \cdot s^2)$. Cardiac work was calculated as the product of cardiac output and LV developed pressure (peak systolic pressure – preload pressure): [volume displaced in 1 min (in m^3)] • [pressure (in Pa)]. Cardiac output (mL/min) was expressed as m^3 as $1mL = 1 \cdot 10^{-6}$ m^3; developed pressure (mmHg) was expressed in Pa as 1mmHg = 133.322 Pa = 133.322 $kg/(m \cdot s^2)$. The final product was presented in units of Joules/min ($J = m^2 \cdot kg/s^2$).

Catheterization A Millar pressure catheter (Millar Instruments, Inc, Houston, TX) was inserted, as described previously[3]. Catheterization was performed in ventilated, open-chest rats by inserting a catheter into the RV via an apical puncture with a 26-gauge needle. RVSP was recorded and analyzed by using PowerLab date acquisition system.

RV Myocytes Isolation Briefly, after the heart was isolated and cannulated, it was perfused with Ca^{2+}-free solution for 6 minutes. Next, the heart was perfused with 25 ml perfusion buffer containing 25 mg collagenase, 7 mg hyaluronidase and 1 mg trypsin inhibitor (Worthington, Lakewood, NJ) for 30 minutes at 37°C. The RV was then minced and the myocytes were isolated by trituration in a glass pipette.

Quantitative RT-PCR Tissue-total RNA was extracted from RV tissues from FHR and FHR+Dichloroacetate groups using the PureLink Micro-to-Midi Total RNA Isolation Kit (Invitrogen, Carlsbad, CA). mRNA levels of PDH-β and PDK4 were measured using the ABI PRISM 7900HT PCR system (Applied Biosystems, Foster City, CA) and normalized to β2 microglobulin mRNA expression. The cycle threshold value (Ct) for each gene was obtained using SDS RQ manager 1.2 (Applied Biosystems, Foster City, CA).

PDH activity The measurement of PDH activity was performed as previously described[4]. Briefly, immunocapturing of PDH was performed with anti-PDH antibody immobilized on a dipstick by following the manufacturer's instructions (Abcam, Cambridge, MA). The colored precipitate was quantified using Image J software (NIH, Bethesda, MD).

Immunostaining After the fixation, frozen sections of RV were incubated with primary antibodies for 1 hour and secondary antibodies for 30 minutes. The antifade reagent (Prolong Gold; Invitrogen, Life Technologies, Grand Island, NY) contained 4',6-

diamidino-2-phenylindole (DAPI) to stain the nuclei of the cells. Images were acquired with the Zeiss LSM 510 META confocal microscope (Carl Zeiss Inc, Thornwood, NY) using 488 nm and 561 nm lasers. DAPI was visualized with the Chameleon Ultra 2 photon laser (Coherent Inc, Santa Clara, CA) at 770 nm. Immunofluorescence intensities were calculated with ImageJ after background subtraction.

Western blotting The protein expression levels of PDK4 and forkhead box protein O1 (FOXO1), a putative regulator of PDK4 expression, were assessed by immunoblot on 20µg of RV protein as described previously[1]. The primary antibodies used were anti-PDK1-4, anti-FOXO1 (Abcam, Cambridge, MA) and anti-actin (Millipore, Billerica, MA). The secondary antibodies were anti-rabbit IgG and anti-mouse IgG antibodies (Cell Signaling Technology, Boston, MA).

Supplemantal Tables:

Supplemental Table 1. Rat glucose metabolism PCR Array profile*

Genes	Description	FHR		FHR+DCA	
		Fold regulation vs. CTR	P values vs. CTR	Fold regulation vs. FHR	P values vs. FHR
Pdk4	Pyruvate dehydrogenase kinase, isozyme 4	6.24	0.0031	-4.18	0.0208
Aldob	Aldolase B, fructose-bisphosphate	4.47	0.0005	-1.68	0.0585
Pgls	6-phosphogluconolactonase	-17.73	0	-1.83	0.0267
Tpi1	Triosephosphate isomerase 1	-47.22	0.00001	-1.18	0.5141
Hk2	Hexokinase 2	-4.17	0.0002	-1.07	0.8297
Pgm3	Phosphoglucomutase 3	-2.67	0.0072	1.95	0.1475
Tkt	Transketolase	-2.67	0.0014	1.42	0.1573
Pgm2	Phosphoglucomutase 2	-2.6	0.0007	1.15	0.6873
Pygl	Phosphorylase, glycogen, liver	-2.46	0.0013	1.2	0.6609
Pdp2	Pyruvate dehyrogenase phosphatase catalytic subunit 2	-2.37	0.0002	1.42	0.1181
Rbks	Ribokinase	-2.12	0.0046	1.3	0.4675
Phkg2	Phosphorylase kinase, gamma 2 (testis)	-2.08	0.0038	1.15	0.4769
Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	-2.04	0.0018	1.23	0.1987

H6pd	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	-2.03	0.0003	-1	0.9602
Fbp1	Fructose-1,6-bisphosphatase 1	1.71	0.0475	-1.41	0.1702
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	1.3	0.0492	1.06	0.7077
Gsk3b	Glycogen synthase kinase 3 beta	-1.92	0.0001	1.1	0.496
Idh3g	Isocitrate dehydrogenase 3 (NAD), gamma	-1.88	0.0012	-1.2	0.2261
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2	-1.86	0.0008	1.21	0.3388
Gsk3a	Glycogen synthase kinase 3 alpha	-1.83	0.0007	1.04	0.8893
Bpgm	2,3-bisphosphoglycerate mutase	-1.83	0.001	1.06	0.7989
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1	-1.8	0.0155	1.1491	0.8241
Pdk3	Pyruvate dehydrogenase kinase, isozyme 3	-1.78	0.003	1.54	0.0768
Ogdhl	Oxoglutarate dehydrogenase-like	-1.73	0.0013	-1.78	0.0177
Rpia	Ribose 5-phosphate isomerase A	-1.7	0.0002	-1.07	0.4764
Acly	ATP citrate lyase	-1.7	0.0173	1.1	0.7586
Pc	Pyruvate carboxylase	-1.63	0.0081	1.22	0.0652
Pfkl	Phosphofructokinase, liver	-1.63	0.029	1.45	0.2611
Sucla2	Succinate-CoA ligase, ADP-forming, beta subunit	-1.66	0.0003	1.12	0.4128
Suclg2	Succinate-CoA ligase, GDP-forming, beta subunit	-1.63	0.0023	-1.04	0.736
Dlst	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	-1.61	0.0045	-1.18	0.39
G6pc3	Glucose 6 phosphatase, catalytic3	-1.58	0.0073	-1.03	0.782
Phkg1	Phosphorylase kinase, gamma 1	-1.55	0.0151	1.41	0.0133
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	-1.51	0.0121	-1.08	0.7374
Gck	Glucokinase	-1.51	0.0478	-1.14	0.5542
Pgm1	Phosphoglucomutase 1	-1.48	0.0002	-1.11	0.4721
Gys1	Glycogen synthase 1, muscle	-1.41	0.0034	1.1	0.4738
Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha	-1.41	0.0023	-1.01	0.8955

Galm	Galactose mutarotase (aldose 1-epimerase)	-1.36	0.0371	1.2	0.197
Pgk1	Phosphoglycerate kinase 1	-1.35	0.0318	-1.02	0.8566
Gpi	Glucose phosphate isomerase	-1.31	0.029	1.04	0.8358
Pdpr	Pyruvate dehydrogenase phosphatase regulatory subunit	-1.31	0.037	1.1	0.5499

The upregulation more than 2 is labeled in red and the downregulation less than -2 is labeled in dark green. P<0.05 is labeled in blue.

*The expression of below 42 glucose metabolism-related genes were not significantly changed both in FHR and FHR+Dichloroacetate groups: aconitase 1, 2 (mitochondrial), amylo-1,6-glucosidase,4-alpha-glucanotransferase, aldolase A, aldolase C, citrate synthase, dihydrolipoamide S-acetyltransferase, dihydrolipoamide dehydrogenase, enolase 1, 2, 3, fructose-1,6-bisphosphatase 2, fumarate hydratase 1, glucose-6-phosphatase (catalytic subunit), glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (spermatogenic), glucan (1,4-alpha-), glycogen synthase 2, hexokinase 3, isocitrate dehydrogenase 1 (NADP+), isocitrate dehydrogenase 2 (NADP+) (mitochondrial), isocitrate dehydrogenase 3 (NAD+) beta, malate dehydrogenase 1, malate dehydrogenase 1B, malate dehydrogenase 2, phosphoenolpyruvate carboxykinase 1 (soluble), pyruvate dehydrogenase (lipoamide) alpha 2, pyruvate dehydrogenase (lipoamide) beta, phosphoglycerate mutase 2 (muscle), phosphoglycerate kinase 2, phosphorylase kinase alpha 1, phosphorylase kinase beta, pyruvate kinase (liver and RBC), phosphoribosyl pyrophosphate synthetase 1, Phosphoribosyl pyrophosphate synthetase 1-like 1, phosphorylase, glycogen (muscle), succinate dehydrogenase complex subunit B iron sulfur (Ip), succinate dehydrogenase complex subunit C (integral membrane protein), succinate dehydrogenase complex subunit D (integral membrane protein), succinate-CoA ligase alpha subunit, transaldolase 1.

Supplemental Table 2. Rat fatty acid metabolism PCR Array profile*

Genes	Description	FHR		FHR+DCA	
		Fold regulation vs. CTR	P values vs. CTR	Fold regulation vs. FHR	P values vs. FHR
Acox2	Acyl-Coenzyme A oxidase 2, branched chain	61.20	0.0000	1.12	0.2373
Acads	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	-2.82	0.0031	1.25	0.6951
Fabp1	Fatty acid binding protein 1, liver	-2.49	0.0005	1.01	0.7572
Acot3	Acyl-CoA thioesterase 3	-2.10	0.0016	1.21	0.3829
Acaa2	Acetyl-Coenzyme A acyltransferase 2	1.75	0.0046	-1.06	0.7816
Fabp3	Fatty acid binding protein 3, muscle and heart	1.56	0.0417	1.04	0.8335
Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	-1.93	0.0002	1.26	0.2375

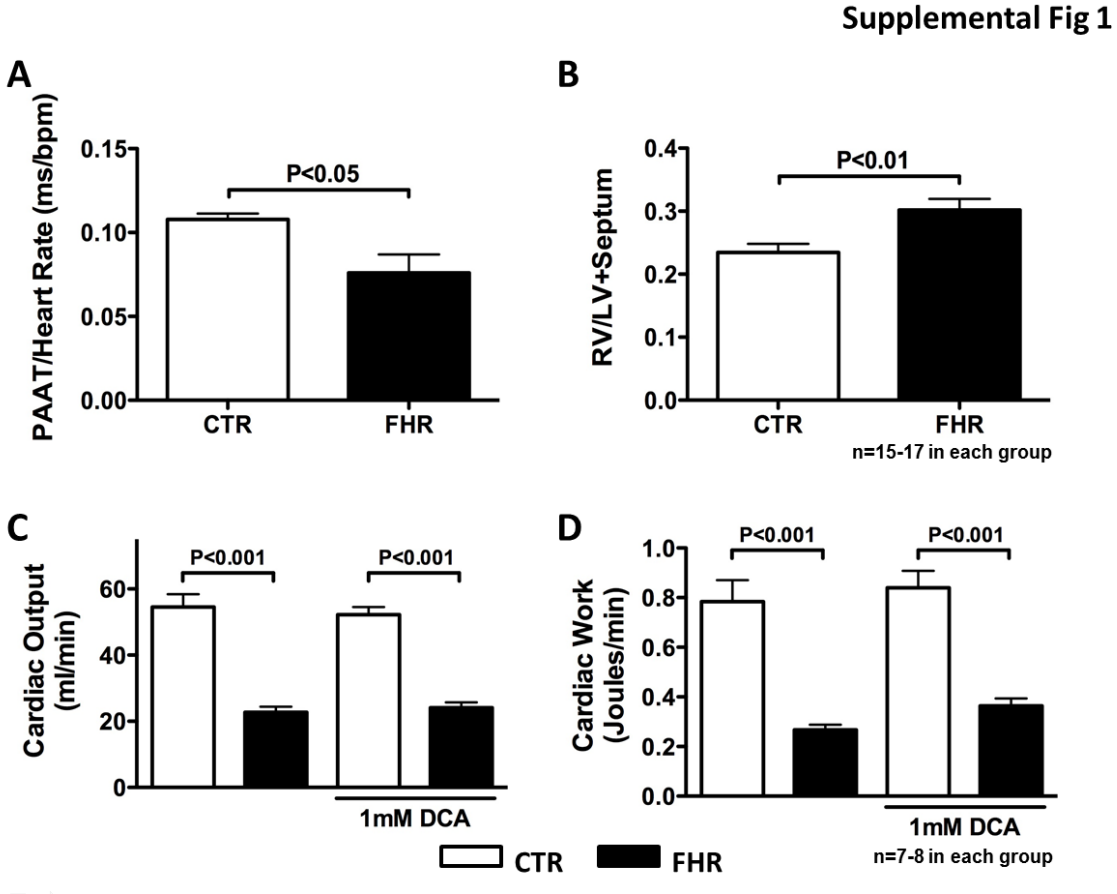
Hmgcl	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	-1.90	0.0083	1.00	0.8511
Slc27a1	Solute carrier family 27 (fatty acid transporter), member 1	-1.88	0.0029	-1.03	0.8843
Acsm3	Acyl-CoA synthetase medium-chain family member 3	-1.81	0.0028	1.08	0.6714
Prkacb	Protein kinase, cAMP dependent, catalytic, beta	-1.75	0.0025	1.19	0.3021
Acadsb	Acyl-Coenzyme A dehydrogenase, short/branched chain	1.70	0.0046	1.01	0.9700
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	-1.67	0.0135	-1.05	0.9915
Echs1	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	-1.64	0.0002	-1.01	0.9605
Prkag2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-1.60	0.0075	1.08	0.6501
Gpd2	Glycerol-3-phosphate dehydrogenase 2, mitochondrial	-1.56	0.0295	1.28	0.2591
Mcee	Methylmalonyl CoA epimerase	-1.56	0.0052	-1.13	0.4943
Acsl1	Acyl-CoA synthetase long-chain family member 1	-1.48	0.0146	1.10	0.5983
Crot	Carnitine O-octanoyltransferase	-1.48	0.0072	1.05	0.6910
Decr2	2,4-dienoyl CoA reductase 2, peroxisomal	-1.42	0.0374	1.04	0.8023
Acot9	Acyl-CoA thioesterase 9	-1.39	0.0227	1.09	0.5192
Prkaca	Protein kinase, cAMP-dependent, catalytic, alpha	-1.38	0.0068	1.11	0.3198
Acsl6	Acyl-CoA synthetase long-chain family member 6	-1.33	0.0459	1.10	0.4790
Bdh2	3-hydroxybutyrate dehydrogenase, type 2	-1.32	0.0396	-1.03	0.7182
Gcdh	Glutaryl-Coenzyme A dehydrogenase	-1.31	0.0052	-1.08	0.3272

Pecr	Peroxisomal trans-2-enoyl-CoA reductase	-1.28	0.0045	1.12	0.2338
Prkag1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	-1.18	0.0179	-1.03	0.5338

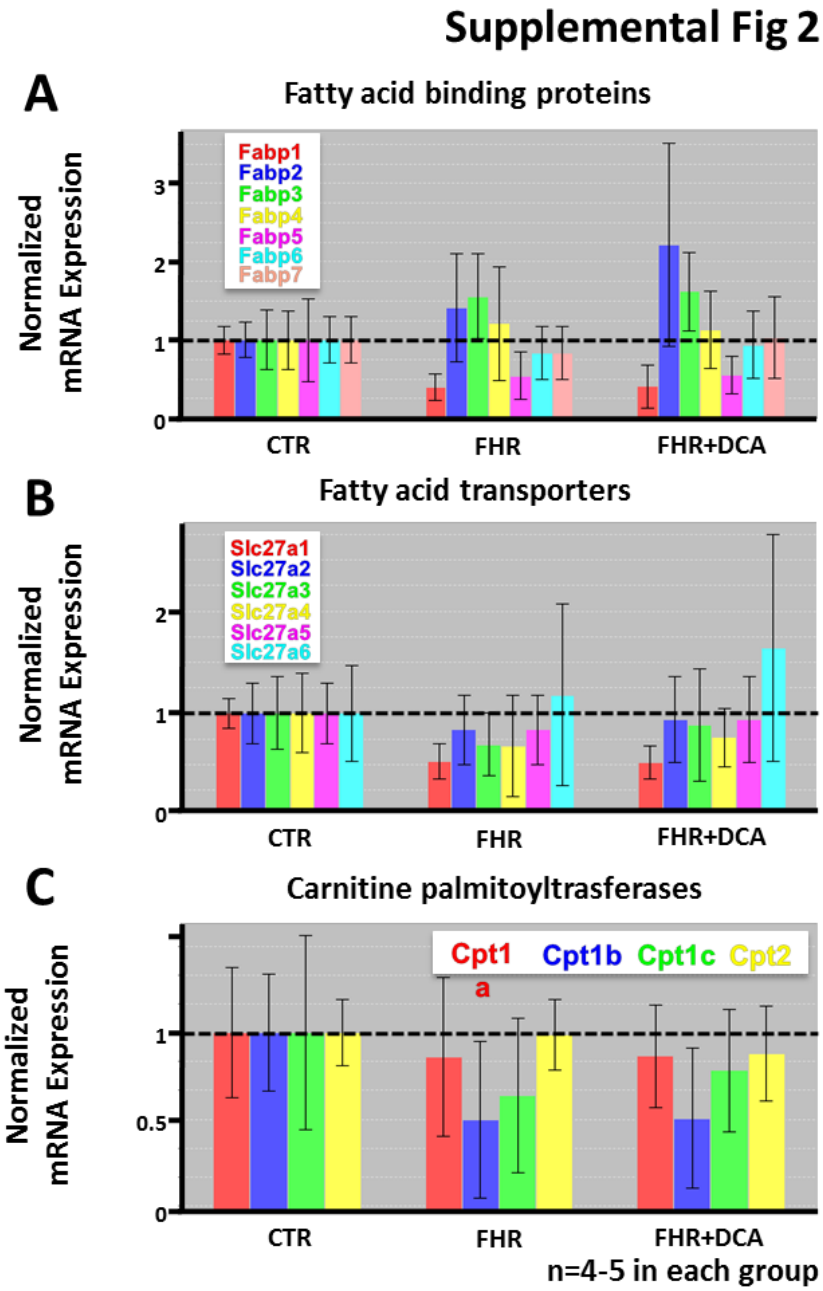
The upregulation more than 2 is labeled in red and the downregulation less than -2 is labeled in dark green. $P<0.05$ is labeled in blue.

*The expression of below 57 fatty acid metabolism-related genes were not significantly changed both in FHR and FHR+Dichloroacetate groups: acetyl-coenzyme A acyltransferase 1A, acyl-coenzyme A dehydrogenase 11, acyl-coenzyme A dehydrogenase 9, long-chain acyl-coenzyme A dehydrogenase, acyl-coenzyme A dhydrogenase (C-4 to C-12 straight chain), acyl-coenzyme A dehydrogenase (very long chain), acetyl-coenzyme A acetyltransferase 1, acetyl-coenzyme A acetyltransferase 3, acyl-CoA thioesterase 12, acyl-CoA thioesterase 2, acyl-CoA thioesterase 7, acyl-CoA thioesterase 8, acyl-coenzyme A oxidase 1, acyl-coenzyme A oxidase 3, acyl-CoA synthetase bubblegum 1, acyl-CoA synthetase bubblegum family member 2, acyl-CoA synthetase long-chain family member 3, acyl-CoA synthetase long-chain family member 4, acyl-CoA synthetase long-chain family member 5, acyl-CoA synthetase medium-chain family member 2, acyl-CoA synthetase medium-chain family member 4, acyl-CoA synthetase medium-chain family member 5, aldehyde dehydrogenase 2 family (mitochondrial), 3-hydroxybutyrate dehydrogenase 1, carnitine palmitoyltransferase 1a, carnitine palmitoyltransferase 1b, carnitine palmitoyltransferase 1c, carnitine palmitoyltransferase 2, carnitine acetyltransferase, 2,4-dienoyl CoA reductase 1(mitochondrial), fatty acid binding protein 2, fatty acid binding protein 4, fatty acid binding protein 5, Fatty acid binding protein 6, Fatty acid binding protein 7, Glycerol kinase, Glycerol kinase 2, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase alpha subunit, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial), hormone sensitive lipase, Lipoprotein lipase, methylmalonyl-Coenzyme A mutase, 3-oxoacid CoA transferase 2A, enoyl-Coenzyme A delta isomerase 2, pyrophosphatase (inorganic) 1, AMP-activated Protein kinase alpha 1, AMP-activated Protein kinase alpha 2, AMP-activated Protein kinase beta 1 subunit, AMP-activated Protein kinase beta 2 subunit, AMP-activated Protein kinase gamma 3 subunit, solute carrier family 27 (fatty acid transporter) member 2, 3, 4, 5, 6.

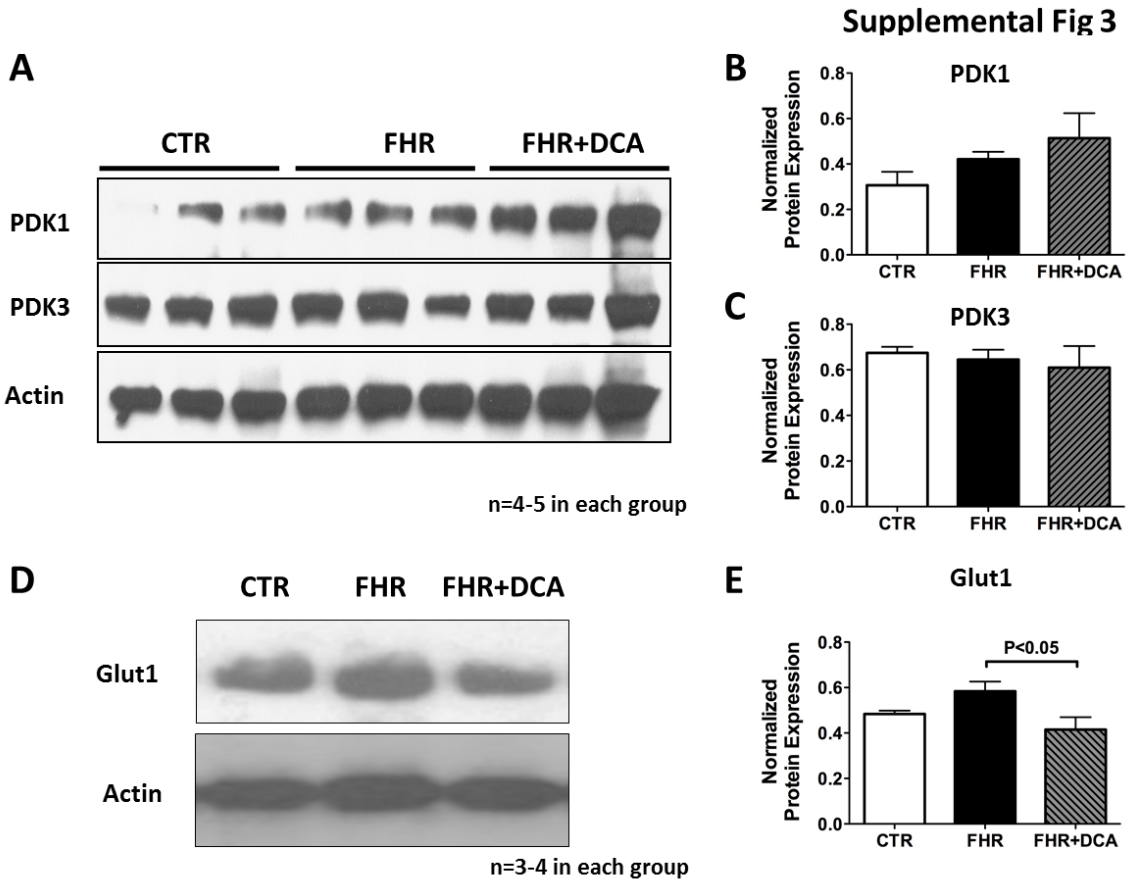
Supplemental Figures:



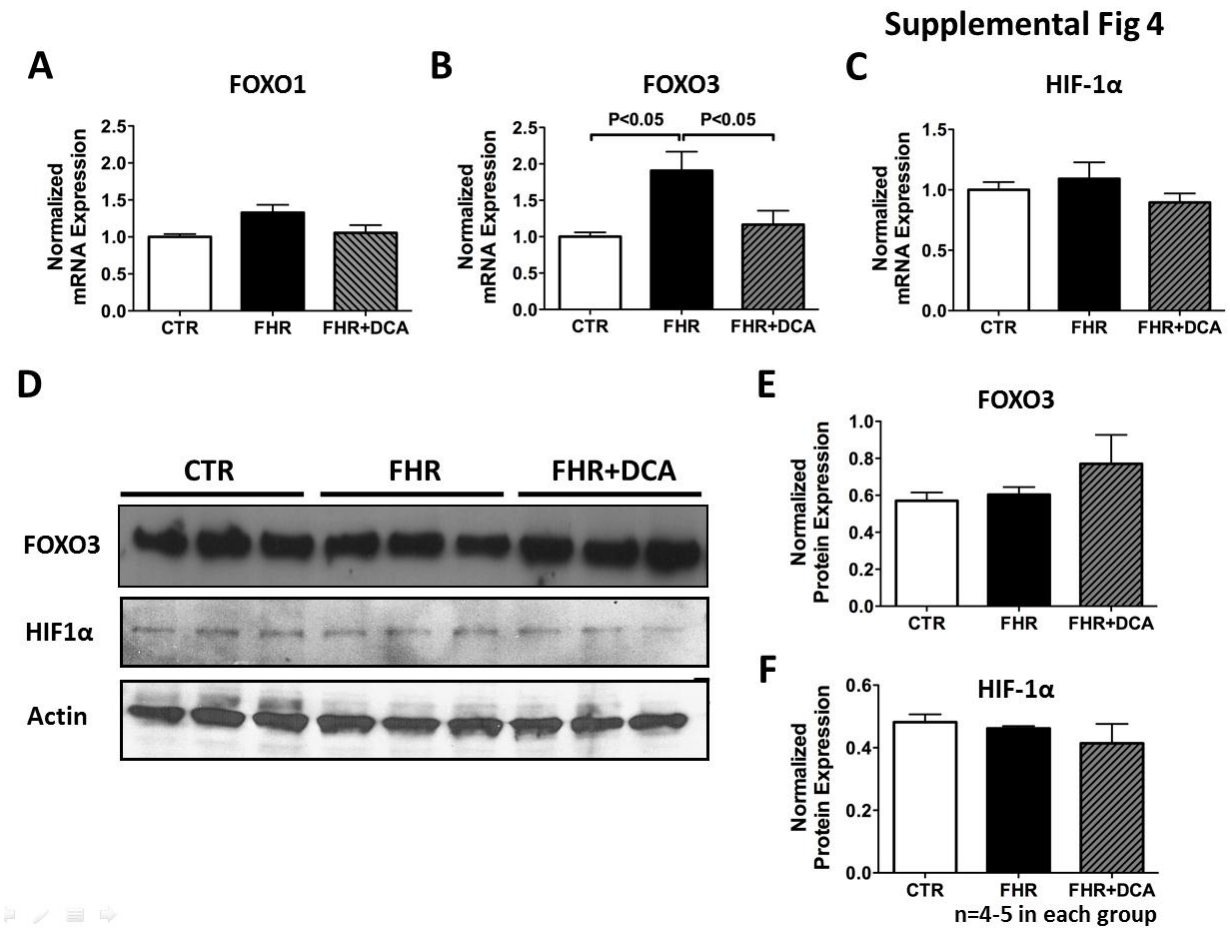
Supplemental Figure 1: Cardiac function is not changed by acute dichloroloacetate in the heart in FHR RVH. Prior to initiation of therapy FHR had evidence of PAH, including **A.** reduced pulmonary artery acceleration time (PAAT) corrected for heart rate (HR). **B.** RVH (increased RV/LV+septum ratio). Cardiac output (**C**) and cardiac work (**D**) are reduced in FHR heart and dichloroacetate does not change both parameters.



Supplemental Figure 2: Fatty acid oxidation-related genes remain unchanged in RV in FHR. Fatty acid binding proteins (**A**), fatty acid transporters (**B**) and carnitine palmitoyltrasferases (**C**) are not changed in RV in FHR and dichloroacetate doesn't alter the expressions.



Supplemental Figure 3: The protein expressions of PDK1, PDK3 and Glut1 in RV in FHR. A-C. Immunobloting bands and bar graphs showing the protein expression of PDK1 and PDK3 are not changed in RV in FHR and dichloroacetate does not alter the proteins expressions. **D-E.** The protein expressions of Glut1 is increased in RV in FHR and reduced by chronic dichloroacetate treatment.



Supplemental Figure 4: The changes of the transcriptional factors in RV in FHR. A. The mRNA expressions of FOXO1 tend to increase in RV in FHR and dichloroacetate tends to decrease the expressions. **B.** The mRNA expressions of FOXO3 are increased in RV in FHR and dichloroacetate normalizes the increase. **C.** The mRNA expressions of HIF-1α are not changed in RV in FHR and dichloroacetate does not alter the expressions. D-F. The protein expression of FOXO3 and HIF-1α remain unchanged in RV in FHR and FHR+dichloroacetate.

References

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