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Supplemental Information

Fatty Acids Enhance the Maturation of Cardiomyocytes Derived from Human Pluripotent Stem Cells

Xiulan Yang, Marita L. Rodriguez, Andrea Leonard, Lihua Sun, Karin A. Fischer, Yuliang Wang, Julia Ritterhoff, Limei Zhao, Stephen C. Kolwicz Jr., Lil Pabon, Hans Reinecke, Nathan J. Sniadecki, Rong Tian, Hannele Ruohola-Baker, Haodong Xu, and Charles E. Murry

SUPPLEMENTAL INFORMATION

Figures and Legends

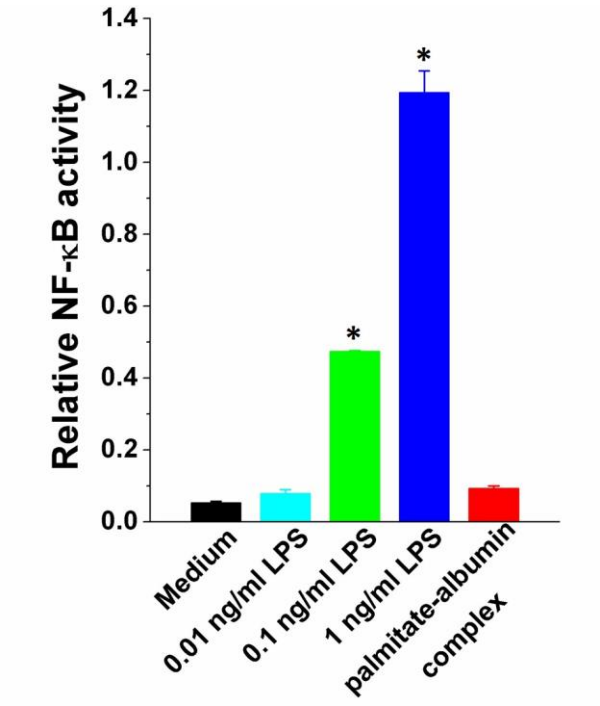

Figure S1. House-made palmitate-albumin complex is endotoxin-free, related to Results 2.1. HEK 293 cells were treated with medium only or medium containing 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml LPS or the self-made palmitate-albumin complexes. No significant endotoxin activity was observed in the medium containing palmitate-albumin complexes compared with medium only. The assay was performed three times on three different batches of palmitate-albumin complex. * $P<0.05$ vs medium only. Data are represened as mean ± SEM.

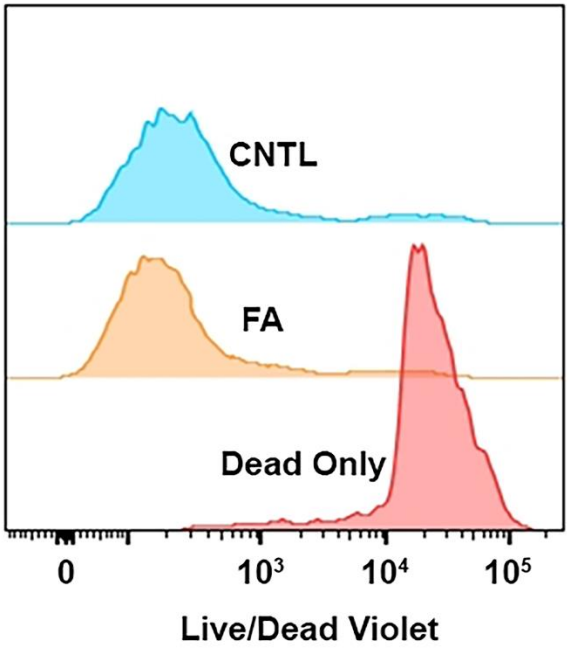

Figure S2. Fatty acids supplement does not lead to cell toxicity, related to Results 2.1. Live-dead assay showed there was no difference in cell death between control and fatty acids-treated hPSC-CMs, two weeks after treatment. The experiments were performed with cardiomyocytes from three different cardiomyocyte differentiation runs and similar results were observed.

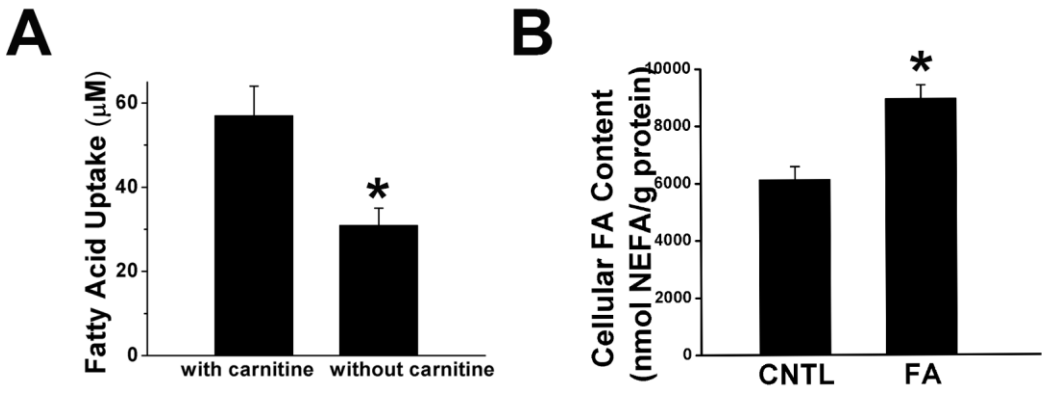

Figure S3. Cellular fatty acids uptake and cellular fatty acid content, related to Results 2.1. Panel A showed cellular fatty acids uptake with or without carnitine added to the medium. B shows the intracellular

fatty acid content with and without FA treatment. Experiments were performed with cardiomyocytes from three different differentiation runs, * P< 0.05 between groups. Data are represented as mean ± SEM.

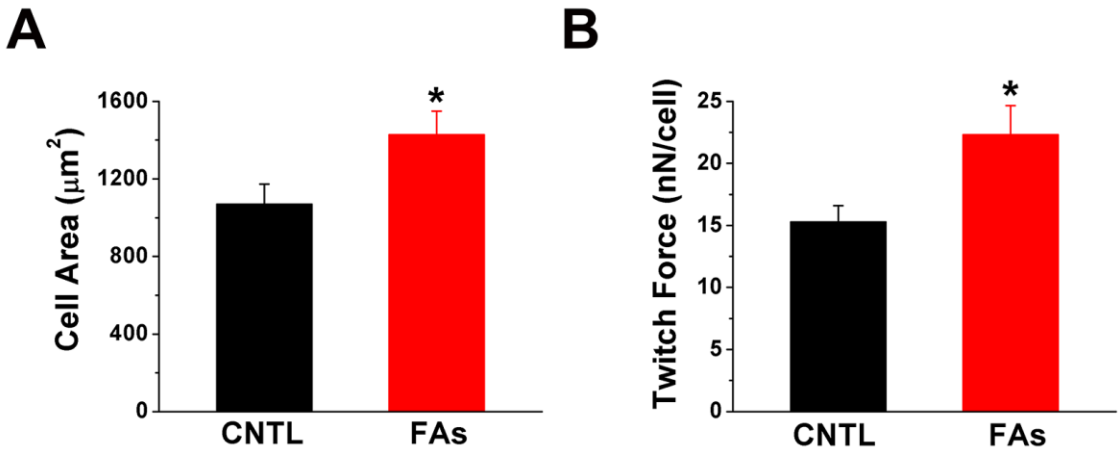

Figure S4. The effect of fatty acids treatment on cardiomyocytes derived from RUES cells, related to Figure 1 and Figure 3. (A) Fatty acids treatment leads to hypertrophy of cardiomyocytes-derived from RUES2 embryonic stem line, n>170 from 3 different cardiomyocyte differentiation runs. Data are represented as mean ± SEM. * P<0.05. (B) Fatty acids treatment significantly increases the contractile force of cardiomyocytes-derived from RUES2 embryonic stem line. n = 26 for control hPSC-CMs and n = 28 for fatty acid-treated hPSC-CMs from 3 different cardiomyocyte differentiation runs. Data are represented as mean ± SEM. * P<0.05 vs. control hPSC-CMs.

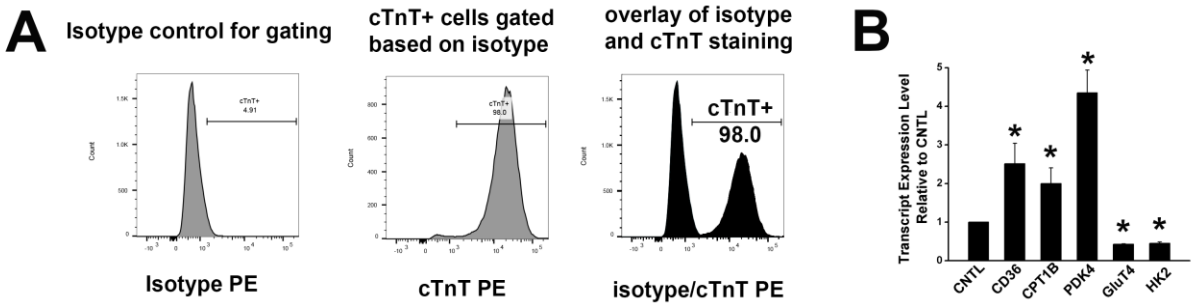

Figure S5. The effect of cardiomyocyte purity on gene expression levels, related to Figure 6. A shows a representative flow cytometry analysis for a cardiac differentiation run which has a cardiomyocyte purity of 98%. B shows Q-PCR assay after treating the high purity cardiomyocytes with fatty acids. The experiments were performed three times with cardiomyocytes from three different differentiation runs. Data are represented as mean ± SEM. * P<0.05 vs. control hPSC-CMs.

Table S1. Primer sequences for Q-PCR assay, related to Figure 6.

Transcripts	**Forward Primer Sequence**	**Reverse Primer Sequence**
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
CD36	TCTTTCCTGCAGCCCAATG	AGCCTCTGTTCCAACTGATAGTGA
CPT1B	ACATCTCTGCCCAAGCTTCC	ACCATGACTTGAGCACCAGG
PDK4	AGAGGTGGAGCATTTCTCGC	ATGTTGGCGAGTCTCACAGG
GluT4	CTCGATCCGACTCGGGAAAG	CGCTGCTGAGGGGGTTC
HK2(Wolf et al., 2011)	CAAAGTGACAGTGGGTGTGG	GCCAGGTCCTTCACTGTCTC

HPRT (hypoxanthine-guanine phosphoribosylthransferase), *CD36* (fatty acid translocase), *CPT1B* (Carnitine palmitoyltransferase 1A), *PDK4* (pyruvate dehydrogenase kinase-4), *GluT4* (glucose transporter 4), *HK2* (hexokinase 2).

Table S2. Electrophysiological parameters for control and FA-treated IMR90-CMs, related to Figure 4.

	CNTL	FA-treated
N	24	25
Beating rate(bpm)	124.45 ± 16.55	115.24 ± 13.20
dV/dt_{max} (V/sec)	23.55 ± 1.80	37.01 ± 5.74*
MDP(mV)	-58.28 ± 1.51	-58.84 ± 1.99
APA(mV)	88.23 ± 3.06	90.69 ± 3.75
APD50 (ms)	255.61 ± 41.49	281.86 ± 35.21
APD90 (ms)	316.33 ± 46.71	343.33 ± 35.52
Cm(pF)	59.56 ± 4.69 (n=14)	73.73 ± 4.60 (n=15)*

N, cell number (from 3 different directed differentiation runs); dV/dt_{max}, maximum rate of action potential upstroke; MDP, maximum diastolic potential; APA indicates action potential amplitude; APD50, action

potential duration measured at 50% repolarization; APD90, action potential duration measured at 90% repolarization; Cm, membrane capacitance; Data were obtained from 3 different cardiomyocyte differentiation runs. For control group n = 24 and n = 25 for FA-treated group. *P<0.05 between control and FA-treated IMR90-CMs.

Supplemental Experimental Procedures

1. Endotoxin assay on the self-made palmitate-albumin complexes

Endotoxin levels in palmitate-albumin complexes were tested by performing HEK293 cell Toll-like Receptor (TLR4) activation assays(Coats et al., 2011). Briefly, HEK293 cells grown in DMEM 10%FBS were plated in 96-well plates at a density of 4 x 104 cells per well, and transfected the following day with plasmids bearing fire-fly luciferase, Renilla luciferase, human HATLR4, MD-2 (lymphocyte antigen 96), and mCD14 by standard calcium phosphate precipitation. Twenty-two hours after transfection, cells were stimulated with the different samples listed in Figure S1, each in triplicate, for 4 hours at 37ºC with the indicated doses and different concentrations of E.coli lipopolysaccharides (LPS) suspended in DMEM/ 5%FBS following stimulation. The transfected HEK293 cells were rinsed with phosphate-buffered saline and lysed with 50 µl of passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). Data are expressed as relative NF-κB-activity, which represents the ratio of NF-κB-dependent fire-fly luciferase activity to β-Actin promoter-dependent Renilla luciferase activity.

2. Flow cytometry live dead assay

The hPSC-CMs were treated with fatty acids for two days and the lipid toxicity was determined by live/dead fixable dead cell stain kit from Invitrogen. The control dead cells were obtained by putting the cells into 60ºC water bath for half an hour.

3. Fatty acid uptake and cellular fatty acid content

The hPSC-CMs were treated with fatty acids for two days and cellular fatty acids uptake was measured using the HR Series NEFA-HR (2) kit from Wako Life Sciences as mentioned in the methods part of the main text. The hPSC-CMs were treated with fatty acids for two weeks and cells were homogenized and FA content was measured as described in the methods part of the main text.

4. Cellular morphology and contractile force changes after FA exposure in cardiomyocytes-derived from RUES2 embryonic stem cells

Methods are similar with the methods with cardiomyocytes-derived from iMR90 cells, please refer to session 2.2, 2.4, 2.5, and 2.8 in the main text for details.

5. Gene expression assay on high purity cardiomyocytes

Q-PCR assay was done in the same way as described in the “Quantitative RT-PCR” part of the main text.

Supplemental Table 1. Primer sequences for Q-PCR assay.

Supplemental Table 2. Electrophysiological parameters for control and FA-treated IMR90-CMs

Results

1. Endotoxin assay on self-made palmitate-albumin complexes

To ensure that endotoxin was not introduced during the process of making palmitate-albumin complexes, endotoxin assay was performed in HEK293 cells. As shown in Figure S1, there was no introduced endotoxin activity in the palmitate-albumin samples, compared to the medium only sample. The same experiment was performed for three different batches of palmitate-albumin complexes and similar endotoxin activity was observed.

2. Live dead assay after fatty acids treatment

Intracellular accumulation of long chain fatty acids in non-adipose tissues is associated with cellular dysfunction and cell death. The toxic effect is mainly mediated by saturated fatty acids, in this study, potentially by palmitate, which may induce apoptosis through both ceramide-dependent(Dyntar et al., 2001) and ceramide-independent(Listenberger et al., 2001) pathways. It has also been reported that palmitate induces cell death through generating reactive oxygen species(Listenberger et al., 2001). Reports also showed that co-administration of unsaturated fatty acids inhibits the pro-apoptotic effects of saturated fatty acids(Miller et al., 2005; Tumova et al., 2015). In this study, both saturated and unsaturated fatty acids were utilized at a physiological ratio and concentration that is present in the newborn serum. Careful observation was made after fatty acids supplement and no cell death was noticed. Live-dead assay was performed after two weeks of fatty acids treatment, similar cell numbers were obtained from the fatty acids treated and untreated groups, and no cell death was detected (Figure S2).

3. Fatty acids uptake and cellular fatty acid content

After two days of feeding, cells take up 57 ± 7 μM of fatty acids with carnitine added. Without adding carnitine, cells take up 31 ± 4 μM of fatty acids (Figure S3A). At the end of the two weeks FA treatment, intracellular fatty acid content was measured and normalized to protein concentration. FA content is significantly increased after FA treatment (6118 ± 461 nmol NEFA/g protein in control vs 8933 ± 488 nmol NEFA/g protein in FA-treated, $n = 3$, $P < 0.05$, Figure S3B).

4. The effect of fatty acids treatment on cardiomyocytes-derived from RUES 2 cell line

In the cardiomyocytes derived from human Rockefeller University Embryonic Stem Cell Line 2, fatty acids treatment induced cellular hypertrophy (1071 ± 102 μm^2 for control cells vs. 1430 ± 119 μm^2 after fatty acids treatment, $P<0.05$, Figure S4A). Also, fatty acids treatment leads to increased contractile force (15.31 ± 1.29 nN/cell for control cells, n=26, vs. 22.33 ± 2.33 nN/cell after fatty acids treatment, n=28, $P<0.05$, Figure S4B).

5. The effect of cardiomyocyte purity on gene expression levels

As mentioned in the methods session, runs that have over 80% cardiomyocyte purity were used in this study, to exclude the effect of ~20% non-cardiomyocytes, high purity cardiomyocytes (over 95%) were used to perform the same Q-PCR assay and similar results was observed (Figure S5).

Supplemental References

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