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***SIRT3* is regulated by nutrient excess and modulates hepatic susceptibility to lipotoxicity**

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Abstract

SIRT3 is the primary mitochondrial deacetylase that modulates mitochondrial metabolic and oxidative stress regulatory pathways. However, its role in response to nutrient excess remains unknown. Thus, we investigated *SIRT3* regulation of the electron transfer chain and evaluated the role of *SIRT3* in hepatic lipotoxic stress. *SIRT3* depleted HepG2 cells shows diffuse disruption in mitochondrial electron transfer chain functioning, a concurrent reduction in the mitochondrial membrane potential, and excess basal reactive oxygen species levels. As this phenotype may predispose to increased lipotoxic hepatic susceptibility we evaluated the expression of *SIRT3* in murine liver following chronic high-fat feeding. In this nutrient-excess model *SIRT3* transcript and protein levels are downregulated in parallel with increased hepatic fat storage and oxidative stress. Palmitate was used to investigate lipotoxic susceptibility in *SIRT3* knockout mouse primary hepatocytes and *SIRT3* siRNA depleted HepG2 cells. Under *SIRT3* deficient conditions palmitate enhances reactive oxygen species and increases hepatocyte cell death. Reconstitution of *SIRT3* levels and/or treatment with N-acetylcysteine ameliorates these adverse effects. In conclusion *SIRT3* functions to ameliorate hepatic lipotoxicity, although paradoxically, exposure to high-fat downregulates this adaptive program in the liver. This *SIRT3*-dependent lipotoxic susceptibility is possibly modulated, in part, by *SIRT3* mediated control of electron transfer chain flux.

Keywords

SIRT3; Lipotoxicity; Electron Transfer Chain; Reactive Oxygen Species; Palmitate

Introduction

Lysine-residue acetylation as a mechanism of post-translation modification and regulation of proteins are evident from prokaryotes to eukaryotes, [1,2] and proteomic screening has identified nutrient-flux dependent changes in the acetylation state of a numerous hepatic mitochondrial metabolic regulatory proteins [1,3]. *SIRT3* is enriched in the liver [4] and is a major mitochondrial NAD-dependent protein deacetylase [5]. Known targets activated by *SIRT3* include complex I and II proteins in the electron transfer chain (ETC), [4,6] and

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proteins that facilitate the generation of tricarboxylic acid (TCA) cycle intermediates [5,7,8]. Whether further components of the electron transfer chain (ETC) are functionally modified by SIRT3 is unknown. Additionally as the acetylation status of the mitochondrial proteome is modified by nutrient flux whether SIRT3 plays a functional role in response to hepatic nutrient stressors warrants investigation.

In this study we investigate the role of SIRT3 in the modulation of mitochondrial electron transfer and the consequences of hepatocyte *SIRT3* deficiency to lipid-mediated toxicity. We show that following *SIRT3* knockdown that complex II initiated respiration in mitochondria *in-situ* is not perturbed, however, complex I and complex IV – V substrate dependent oxygen consumption is significantly blunted compared to controls. In parallel, *SIRT3* depletion results in a reduction in the mitochondrial membrane potential with increased reactive oxygen species (ROS) levels. N-acetylcysteine (NAC) administration reverses the increased ROS levels. As a functional correlation, hepatic tolerance to palmitate is diminished in parallel with palmitate mediated induction of ROS. This lipotoxic susceptibility is also reversed by the reconstitution of SIRT3 to knockout primary hepatocytes. Together these data show that SIRT3 is integral for global ETC functioning and its depletion results in diminished mitochondrial oxygen consumption, excess reactive oxygen levels and enhanced susceptibility to palmitate-mediated hepatocyte cell death.

Materials and Methods

Cell cultures and transfections

HepG2 human hepatocyte cell line was from American Type Cell Culture (ATCC, Manassas, VA) and was maintained in DMEM containing 25mM glucose and 10% fetal bovine serum (FBS). Primary mouse hepatocytes and mouse embryonic fibroblasts were isolated and cultured as described previously [9,10]. For siRNA transfection, 10⁶ HepG2 cells were electroporated with 100nmol of SIRT3 or control ON-TARGET plus SMARTpool siRNA (Thermoscientific) according to the manufacturer's instruction (Amaxa). Unless specified, all the experiments were performed 64–68 hours after transfection. For plasmid transfection, mouse primary hepatocytes were transfected with pcDNA3.1(+) (Invitrogen) or pcDNA3.1 (+) containing full length human SIRT3 cDNA (hSIRT3, Addgene) at 2μg DNA/5μl lipofectamine 2000 reagent in a 6-well type I collagen-coated culture plate. The cells are harvested 48 hours after transfection for further experiments.

Cellular oxygen consumption assay

Steady state cell respiration in HepG2 cells and primary hepatocytes were measured in non-buffered DMEM containing 5.5 mM glucose for HepG2 cells or 10 mM glucose for hepatocytes with XF24 analyzer (Seahorse Bioscience) according to the manual. To examine mitochondrial complex activities, HepG2 cells were permeabilized with 10μg digitonin/10⁶ cells, and incubated with the medium containing 250mM sucrose, 2 mM KH₂PO₄, 10mMMgCl₂, 0.5 mM EGTA, 0.1% fatty acid free BSA, ADP 2mM, 20mM HEPES, pH7.1 in a non-CO₂ incubator for 1 hour before experiments. The cells were then subjected to three to four baseline measurement, followed by injection of the following reagents: 10mM glutamate/5 mM malate for complex I activity, 0.1 μM rotenone/10 mM succinate for complex II activity, antimycin 20nM/0.5 mM TMPD/2 mM ascorbate for complex IV+V activities.

ATP production assay

Steady state cellular ATP levels were measured by using ATP bioluminescence assay kit CLS II in accordance with the protocol (Roche).

Determination of reactive oxygen species (ROS) production

Cellular ROS production was detected with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Invitrogen). Briefly, 10⁶ cells were incubated with 5 μ M CM-H₂DCFDA in serum free medium at 37°C for 20min, after three wash with PBS, the cells were suspended with 100 μ l of PBS and transferred into a 96 well plate with black walls, measured with a Magellan microplate reader (Tecan) every 2 min for 1 hour at 37°C. The excitation and emission wave length are 492nm, and 525nm respectively. In some experiments, the cells were pre-incubated with 10 mM NAC for 1 hour before staining with CM-H₂DCFDA. In other experiments, 0.5 mM palmitate/0.5% fatty acid free BSA or 0.5% BSA were added to the cell suspension immediately before measurement. ROS production was calculated as the maximal slope (RFU/min) in each reaction. Mitochondrial ROS production was additionally measured using Mitosox (Invitrogen), following the same protocol for CM-H₂DCFDA above. After incubation with 1 μ M Mitosox for 20 min, 10⁶ cells were analyzed on the plate reader using an excitation and emission wavelength of 492 nm and 595 nm, respectively.

Immunoblot analysis and immunoprecipitation

HepG2 cells or mouse liver tissues were extracted in RIPA lysis buffer and 20 μ g of total proteins were separated in 4–20% tris-glycine gel. After transferred into nitrocellulose membranes, the proteins were blotted with the following primary antibodies: rabbit polyclonal anti-human SIRT3 antibody (Cell Signaling), affinity-purified rabbit polyclonal anti-SIRT3 antibody was raised against the 15 amino acids of the mouse SIRT3 C-terminus [5], monoclonal anti-alpha tubulin antibody (Santa Cruz) and mitochondrial F1F0-ATPase subunit α (Invitrogen).

For immunoprecipitation, liver mitochondrial proteins from WT and SIRT3 KO mice were purified by differential centrifuge as described [11], and 1mg mitochondrial proteins were solubilized with 1% n-dodecyl- β -D-maltopyranoside and mitochondrial complex V components were precipitated with ATPase synthase immunocapture kit (MitoSciences), followed by immunoblotting using the following antibodies: affinity-purified rabbit polyclonal anti-SIRT3 antibody was raised against the 15 peptides of mouse SIRT3 C-terminus, rabbit polyclonal anti-acetylated lysine antibody was from Cell Signaling, and F1F0-ATPase subunit α as described above. To assess human SIRT3 levels the Sigma antibody was used. Immunoblots were visualized and analyzed using the Odyssey infrared imaging system (Li-Cor Biosciences).

Gene expression analysis

Following isolation and quantification of RNA from liver extracted from mice fed for 12 weeks on a caloric equivalent chow diet (% calories – carbohydrates - 70, protein – 20 and fat - 10) versus high fat (% calories – carbohydrates - 20, protein - 20 and fat - 60 (Research Diets Inc.)). The reverse transcription reaction was performed using Super-Script III reverse transcriptase kit (Invitrogen). Real time quantitative-PCR was performed using SYBR green PCR Master Mix (Applied Biosystems) and MJ Research DNA Engine Opticon 2 fluorescence detection system (BioRad). The murine QuantiTect SIRT3 primers were obtained from Qiagen. The mRNA concentration was calculated from the cycle threshold values using a standard curve and normalized to the expression levels of 18S ribosomal RNA.

Liver confocal microscopy

For nitrotyrosine staining frozen liver sections were fixed with methanol at –20°C for 15min and permeabilized with 0.1% Triton X-100 in PBS for 5min at room temperature. After three

wash with PBS, the slides were blocked with 10% normal goat serum for 30min at room temperature, followed by incubation with anti-nitrotyrosine antibody (1:100, Upstate) at 4°C over night. The slides were then incubated with Alexa Fluor 594-conjugated goat antimouseIgG (1:250; Invitrogen) at room temperature for one hour. Nuclei were counterstained with 4'6'-diamidino-2 phenylindole (DAPI; Invitrogen). The images were collected using a Zeiss LSM 510 confocal microscope.

Lipotoxicity assay

To induce lipotoxicity, 0.5mM or 0.75 mM palmitate conjugated to 0.5% fatty acid free BSA or BSA alone was added to HepG2 cells or primary hepatocytes in the presence or absence of 10 mM NAC for 24 hours. Then the cells were harvested and stained with propidium iodide (PI) dye, then subjected to FACS analysis to detect cell viability. The lipotoxicity was calculated as the percentage of PI positive cells (dead cells) in a cell population.

Statistical Analysis

Differences between data groups were evaluated for significance using the Student *t*-test. *P* < 0.05 was considered statistically significant, and data are expressed as mean ± SEM.

Results

SIRT3 regulates mitochondrial respiration via modulation of multiple complexes in the electron transfer chain

To investigate modulation of the mitochondrial ETC by SIRT3, we employed siRNA to deplete *SIRT3* in HepG2 cells (Fig. 1a) and transient transfection to restore SIRT3 levels into the *SIRT3* knockout primary hepatocytes (Fig. 1b). Consistent with prior studies in *SIRT3*^{-/-} mouse embryonic fibroblasts cellular oxygen consumption and ATP levels were significantly reduced compared to levels in scrambled siRNA transfected cells (Fig. 1c–d, *p* < 0.001). Interestingly, the reduction in oxygen consumption is also evident in *SIRT3* knockout primary hepatocytes. The role of SIRT3 in this phenotype is confirmed by the restoration of oxygen consumption following the reintroduction of *SIRT3* into primary *SIRT3*^{-/-} hepatocytes (Fig. 1c). In parallel with these mitochondrial bioenergetic perturbations, the inner mitochondrial membrane potential was diminished following the knockdown of *SIRT3* (Fig. 1e) with a parallel increase in ROS levels (Fig. 1f).

As protein components of complex I, II and V and cytochrome C of the electron transfer chain proteins have been shown to undergo lysine residue acetylation [1,8,12] we delineated individual electron transfer chain complex specific substrate capacity to facilitate oxygen consumption following RNAi knockdown of *SIRT3*. HepG2 cells were permeabilized and complex I, II and IV/V substrates were administered. As shown in Figure 2, the capacity to enhance oxygen consumption was significantly blunted following the supply of substrate feeding into complexes I, III and IV (Fig. 2a–b) and downstream of complex III (Fig. 2e–f) in the *SIRT3* depleted cells compared to scramble siRNA controls (*P* < 0.001). Conversely, although basal respiration was lower prior to the administration of succinate to supply reducing equivalents to complex II in the *SIRT3* knockdown cells, the relative enhancement of oxygen consumption by complex II was similar in the *SIRT3* knockdown and control cells (Fig. 2c–d). It is interesting to note, that while global reduction in oxygen consumption in the absence of SIRT3 is ≈30% (Fig. 1c), and consistent with prior publications [4,10], when interrogating the different complex substrates (Fig. 2), we find much larger differences in baseline oxygen consumption. We propose that as the knockdown of *SIRT3* is known to result in lower ETC complex I and II activity [4,6], the incubation of the permeabilized cells with 2mM ADP (see methods section) in the presence of endogenous metabolites and

reducing equivalents may exaggerate the basal differences in oxygen consumption prior to the experimental introduction of the ETC complex specific substrates. Although this approach does appear to accentuate differences when interrogating the distinct ETC complexes, it should be recognized that these differential oxygen consumption rates do not reflect the composite oxygen consumption as shown in intact cells.

As the acetylation status of complex V is modulated by the manipulation of SIRT3 [8,12], we investigated whether SIRT3 interacts with, and deacetylates, subunits of complex V. This potential function of SIRT3 is supported by protein interaction studies and the differential acetylation levels of the complex V subunit α in wildtype versus *SIRT3* knockout MEF cells (supplemental section Fig. S1a–c).

SIRT3 levels are downregulated in response to a high fat diet

Disrupted mitochondrial function has been shown to result in a predisposition or exacerbation of numerous pathologies including, for example, premature aging, insulin resistance and hepatic lipotoxicity [13–15]. As the liver is susceptible to fat-induced toxicity, [16] we reasoned that it would be important to evaluate the role of SIRT3 in mitochondrial homeostasis in response to this nutrient-excess mediated stress. We began by delineating whether SIRT3 is modulated in the liver in response to chronic fat feeding. Following 12 weeks of a diet with a 60% fat content the mouse liver showed evidence of increased lipid deposits (Fig. 3a) and evidence of redox stress (Fig. 3b). In parallel transcripts encoding for *SIRT3* and steady-state protein levels were significantly downregulated in wild type mice compared to chow-fed controls (Fig 3c–d).

SIRT3 depletion increases ROS levels and enhances susceptibility to lipotoxicity

As a high-fat diet downregulates SIRT3 and the depletion of SIRT3 disrupts mitochondrial function, we investigated whether exposure of hepatocytes to the saturated fatty acid palmitate in the presence of normal or diminished levels of SIRT3 would modulate mitochondrial respiration and ROS levels. Interestingly, the acute exposure to palmitate does increase oxygen consumption compared to BSA vehicle administration in both scrambled and *SIRT3* siRNA transfected HepG2 cells. Although, in parallel with basal respiration, the palmitate-mediated increase in oxygen consumption is lower following SIRT3 depletion (Fig. 4a). In parallel, ROS levels as measured by DCF fluorescence are disproportionately induced by palmitate when SIRT3 levels are diminished (Fig 4b) or in *SIRT3* knockout primary hepatocytes (Fig 4c). The excess palmitate-induced ROS levels evident in *SIRT3* depleted cells are rescued by the co-administration of the anti-oxidant NAC (Fig. 4d). As saturated fatty acids and excess ROS can predispose to lipid peroxidation and cellular injury, we evaluated the response of these cells to increasing concentrations of palmitate administration versus BSA administration. 0.5 mM palmitate increased cell death by $\approx$ 80% in the control siRNA cells and by 150% in the *SIRT3* depleted HepG2 cells (Fig. 5a). 0.75 mM of palmitate further increased cell death in the control and *SIRT3* knockdown cells although demonstrating a continued disadvantage of *SIRT3* depletion (Fig. 5a). In parallel with the ROS findings, the co-administration of NAC reversed palmitate-induced cell death in the *SIRT3* depleted HepG2 cells.

To confirm the role of SIRT3 in this adverse phenotype we explored palmitate-tolerance and the response to *SIRT3* repletion in primary *SIRT3*^{−/−} mouse hepatocytes compared to wild-type controls. As in the siRNA studies, basal cell viability was unchanged comparing wild-type and *SIRT3*^{−/−} hepatocytes (Fig. 5c). 0.5 mM palmitate significantly increased cell death in the *SIRT3*^{−/−} hepatocytes compared to the response in wildtype hepatocytes (Fig. 5c). The restoration of SIRT3 levels in the *SIRT3*^{−/−} hepatocytes following transient

transfection rescued this detrimental phenotype with the return of palmitate-exposed cell viability to similar levels of the wild-type controls (Fig. 5d).

Discussion

Similar to other sirtuins, the mitochondrial enriched SIRT3 is activated by caloric restriction and fasting [5]. The functional targets of SIRT3 support its regulatory role in mitochondrial bioenergetics [4,6,10] and SIRT3 levels are enriched in the liver [4], an important nutrient homeostasis organ. Thus, we reasoned that the delineation of the role of SIRT3 in nutrient-excess mediated hepatic pathology is important to further delineate nutrient-dependent SIRT3 functioning. The major findings presented in this manuscript identify: i) that novel electron transfer sites are regulated by SIRT3; ii) that SIRT3 is downregulated in the liver in response to a high-fat diet, and iii) that the depletion of *SIRT3* exacerbated lipotoxicity via the modulation of ROS.

Mitochondrial metabolism and redox stress proteins have been shown to undergo deacetylation in response to fasting in the liver [1]. However, whether SIRT3 is the deacetylase that orchestrates these effects is predominantly unknown and the functional effect of known targets of SIRT3 deacetylation are only beginning to be explored (reviewed [17]). The known targets of SIRT3 deacetylation modulate substrate entry into the TCA cycle and regulate proximal subunits in the ETC. Recently proteomic analysis implicates ATP synthase alpha subunit of the ETC as a probably additional target of SIRT3 deacetylation [12]. Our functional *in vivo* oxygen consumption studies support that components of the ETC downstream of complex III are modulated and subsequently activated by SIRT3. In addition, we show that numerous subunits of complex V are deacetylated in wild-type compared to *SIRT3* knockout mice liver tissue, and that one or more ATP synthase subunits directly interacts with SIRT3. Additionally, we demonstrate that ATP synthase subunit α shows a higher level of acetylation in *SIRT3* deficient compared to wildtype MEF cells. Although these data support the ATPase subunit α as a substrate of SIRT3, further work will be required to fully delineate the extent of the functional interaction between these two proteins. Interestingly, we show that complex II of the ETC is not directly modulated by SIRT3, which although compatible with the findings of Ahn et al [4], differs from the observations of Cimen et al [6]. This discrepancy is currently unexplained but warrants further investigation.

A primary method of post-translational modification and signaling in mitochondria appears to be via a change in protein acetylation, and SIRT3 is one of the primary protein deacetylases. SIRT3 is activated, at least in part, by changes in the ratio of NAD^+/NADH and SIRT3 deacetylase activity may play a role in metabolic diseases such as obesity or diabetes since these conditions are associated with a diminished NAD^+/NADH ratio and lower NADH oxidase activity [18,19]. Interestingly, a recent study has shown that SIRT3 activates a pivotal mitochondrial fatty acid oxidation enzyme [20]. This observation would further suggest that SIRT3 may play an adaptive role in handling excess fat in the diet whereby it could facilitate the catabolism of these nutrients.

Chronic liver damage is also associated with a reduction in NAD levels [21]. In this regard, our study shows that chronic high-fat feeding increases hepatosteatosis and oxidative liver damage in parallel with the downregulation of SIRT3 levels. We therefore postulated that the mitochondrial dysfunction associated with SIRT3 downregulation or depletion may exacerbate injury in response to lipotoxic insults. The plausibility of this postulate is further supported in that lipotoxicity in the liver is associated with mitochondrial dysfunction [15] and saturated fats *per se* increase mitochondrial ROS levels [22]. In this study we demonstrate that the *SIRT3* knockdown enhances ROS levels and that the ROS scavenger

NAC, reverses this *SIRT3* depletion effect. In parallel, *SIRT3* depletion enhanced susceptibility to palmitate toxicity and this injury is reversed by both NAC and the reconstitution of *SIRT3*. Taken together, these results suggest that the downregulation of *SIRT3* in the liver responding to excess fatty acids would be a maladaptive response and might play a role in hepatic pathophysiology in obesity.

Interestingly, *SIRT3* deficiency has previously been shown to increase susceptibility to direct oxidative stress and in facilitating ROS-dependent oncogenic transformation [23,24]. Mechanisms identified through which the reduction or absence of *SIRT3* mediates these ROS-dependent effects include the disruption of anti-apoptotic programs [23] and via the attenuation of anti-oxidant defense programs [24]. Whether the direct disruption of ETC functioning with the diminution in *SIRT3* is an additional mechanism enabling excess ROS generation, resulting in increased susceptibility to biological stressors, is suggested by the palmitate-mediated adverse effects in this study. However, as additional targets of *SIRT3* deacetylation are being identified, it is increasingly being recognized that the number of targets that may be operational in the control of the mitochondrial and cellular redox state are probably multi-factorial [17].

Although the predominant focus of investigation into *SIRT3* has been studied under conditions that mimic caloric restriction, the reduction in its function, under nutrient excess conditions, may play a similarly important biological role. Excess fat in the diet gives rise to both diabetes and obesity and both of these conditions can give rise to non-alcoholic fatty liver disease. Finally, we expand the targets of *SIRT3* to include the modulation of distal ETC complexes and confirm that the reduction in *SIRT3* levels results in excess ROS levels that is further accentuated in the presence of palmitate. An interesting question arising from this research is whether the retardation of ETC flux with low or absent *SIRT3* levels is a significant contributor to the cytotoxic effects of excess ROS generation. The complexity of dissecting this possible pathophysiology from the myriad of additional yet, uncharacterized targets of *SIRT3* is an important challenge in understanding the full scope of the functioning of *SIRT3*. The importance of the delineation of this biology is, however, underscored by the prevalence of nutrient excess in society, the increasing pathology associated with nutrient excesses and the potential that the regulation of *SIRT3* may play a role in ameliorating these pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SIRT3 regulates mitochondrial functions in hepatic cells

(A) Immunoblot analysis of SIRT3 expression levels in HepG2 cells transfected with control or SIRT3 siRNA for 72 hours. F₀F₁-ATPase subunit α was used as loading controls. (B) Immunoblot analysis shows the absence of SIRT3 in primary knockout (KO) hepatocytes and the capacity to reconstitute SIRT3 levels following transfection studies. (C) Intact cellular basal oxygen consumption rates (OCR) of control or SIRT3 siRNA-treated HepG2 cells, wild type (WT) or SIRT3 KO hepatocytes, and SIRT3 KO hepatocytes transfected with pcDNA3.1 or plasmid encoding hSIRT3 measured by Seahorse XF24 analyzer. (D) Steady-state cellular ATP levels in control or SIRT3 siRNA-treated HepG2 cells. Data represent mean $\pm$ SEM from four independent experiments. (E) Comparison of mitochondrial membrane potential in control or SIRT3 siRNA-treated HepG2 cells, as measured by TMRM. (F) Representative traces of ROS production as indicated by DCF fluorescence intensity in control or SIRT3 siRNA-treated HepG2 cells.

Figure 2. SIRT3 regulates mitochondrial electron transfer chain activities

Left, representative traces of OCRs in control or SIRT3 siRNA-treated, digitonin-permealized HepG2 cells follow the addition of glutamate/malate (A), succinate (C), or TMPD/ascorbate (E). The quantification of OCR change in each group was shown in the right of respective figures. B, D, and F, Data represent average of 10 replicates from one plate $\pm$ SD, each experiment repeated at least three times. **, $p < 0.01$.

Figure 3. Effects of long term high fat diet on hepatic SIRT3 expression and oxidative stress (A) Representative images of oil red O staining of liver sections from mice fed with normal diet or high fat diet for four months. (B) Nitrotyrosine staining (red) of liver sections as described in A. The nuclei were counterstained with DAPI (blue). (C) Liver SIRT3 mRNA expression levels in four month chow diet or high fat diet-fed mice, as quantified by qPCR. Data represent mean $\pm$ SEM, $n = 5$ for chow diet, and $n = 8$ for high fat diet-fed mice. **, $p < 0.01$. (D) Immunoblot analysis of SIRT3 protein expression levels in mouse liver total proteins as described above. β -tubulin was used as loading controls.

Figure 4. A. Palmitate modulates oxygen consumption and ROS levels in a *SIRT3*-dependent manner

(A) Oxygen consumption of control or *SIRT3* siRNA-treated HepG2 cells in response to 0.1 % BSA or 0.1 mM palmitate (PA). Data represent average of 5 replicates from one plate $\pm$ SD, each experiment repeated at least three times. (B) Mitochondrial superoxide production of control or *SIRT3* siRNA-treated HepG2 cells in the absence or presence of 0.5 % BSA or 0.5 mM PA. Data mean $\pm$ SEM from 10 measurements and are representative of at least 3 independent experiments. (C) ROS production in WT and *SIRT3* KO primary hepatocytes in the absence and presence of 0.5 mM PA. The data were expressed as the percentage of maximal slope compared to WT control cells. Data represent mean $\pm$ SEM from three independent experiments. (D) ROS production in control or *SIRT3* siRNA-treated HepG2 cells in the absence or presence of 0.5 mM PA and/or 10 mM NAC. The data were expressed as the percentage of maximal slope compared to control siRNA-treated HepG2 cells. Data represent mean $\pm$ SEM from four independent experiments. *, $p < 0.05$, **, $p < 0.01$.

Figure 5. Oxidative stress partially mediates SIRT3-dependent lipotoxicity

(A) Cell viability of control or SIRT3 siRNA-transfected HepG2 cells following treatment of BSA or different amounts of palmitate (PA). The dead cells are identified as propidium iodide (PI)-positive cells and quantified by FACS analysis. Data represent mean $\pm$ SEM of four independent experiments. (B) Cell viability of control or SIRT3 siRNA-treated HepG2 cells with or without pretreatment of 10mM NAC, followed by incubation with 0.5 mM PA for 24 hours. Data represent mean $\pm$ SEM from four independent experiments. (C) Cell viability of primary WT and SIRT3 KO hepatocytes following treatment of BSA or 0.5 mM palmitate (PA). (D) Cell viability of pcDNA3.1 or hSIRT3 cDNA-transfected SIRT3 KO hepatocytes treated with 0.5 mM PA. Data represent mean $\pm$ SEM of three independent primary cell studies for each group in C and D. A–D, **, $p < 0.01$.