Mitochondrial Reactive Oxygen Species in Lipotoxic Hearts Induce Post-Translational Modifications of AKAP121, DRP1, and OPA1 That Promote Mitochondrial Fission


Rationale: Cardiac lipotoxicity, characterized by increased uptake, oxidation, and accumulation of lipid intermediates, contributes to cardiac dysfunction in obesity and diabetes mellitus. However, mechanisms linking lipid overload and mitochondrial dysfunction are incompletely understood.

Objective: To elucidate the mechanisms for mitochondrial adaptations to lipid overload in postnatal hearts in vivo.

Methods and Results: Using a transgenic mouse model of cardiac lipotoxicity overexpressing ACSL1 (long-chain acyl-CoA synthetase 1) in cardiomyocytes, we show that modestly increased myocardial fatty acid uptake leads to mitochondrial structural remodeling with significant reduction in minimum diameter. This is associated with increased palmitoyl-carnitine oxidation and increased reactive oxygen species (ROS) generation in isolated mitochondria. Mitochondrial morphological changes and elevated ROS generation are also observed in palmitate-treated neonatal rat ventricular cardiomyocytes. Palmitate exposure to neonatal rat ventricular cardiomyocytes initially activates mitochondrial respiration, coupled with increased mitochondrial polarization and ATP synthesis. However, long-term exposure to palmitate (>8 hours) enhances ROS generation, which is accompanied by loss of the mitochondrial reticulum and a pattern suggesting increased mitochondrial fission. Mechanistically, lipid-induced changes in mitochondrial redox status increased mitochondrial fission by increased ubiquitination of AKAP121 (A-kinase anchor protein 121) leading to reduced phosphorylation of DRP1 (dynamin-related protein 1) at Ser637 and altered proteolytic processing of OPA1 (optic atrophy 1). Scavenging mitochondrial ROS restored mitochondrial morphology in vivo and in vitro.

Conclusions: Our results reveal a molecular mechanism by which lipid overload-induced mitochondrial ROS generation causes mitochondrial dysfunction by inducing post-translational modifications of mitochondrial proteins that regulate mitochondrial dynamics. These findings provide a novel mechanism for mitochondrial dysfunction in lipotoxic cardiomyopathy. (Circ Res. 2018;122:58-73. DOI: 10.1161/CIRCRESAHA.117.311307.)

Key Words: heart failure ■ metabolism ■ mitochondrial dynamics ■ oxidative stress ■ reactive oxygen species

To maintain high energy requirements for contractile function, the heart requires an uninterrupted delivery of oxygen and substrates that are oxidized to promote ATP synthesis. ATP consumption primarily fuels sarcomere contraction and ionic pumps that maintain membrane potential.1 In the healthy well-oxygenated heart, mitochondria are central to cardiomyocyte energy metabolism.1 Mitochondrial oxidation of fatty acids (FAs), glucose, ketone bodies, and lactate accounts for the majority of

Original received May 9, 2017; revision received October 25, 2017; accepted October 31, 2017. In September 2017, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13 days.

From the Division of Endocrinology and Metabolism, Fraternal Order of Eagles Diabetes Research Center, Roy J. and Lucille A. Carver College of Medicine (K.T., J.S., G.A.J., A.R.T., R.M., H.C.K., Y.Z., R.S., R.O.P., E.D.A.) and Department of Health and Human Physiology (V.A.L.), University of Iowa, Iowa City; Division of Endocrinology, Metabolism, and Diabetes and Program in Molecular Medicine (K.T., H.B., A.R.W., J.S., X.X.H., C.L.S., E.D.A.), Nora Eccles Harrison Cardiovascular Research and Training Institute (K.W.S.), and Department of Biochemistry (O.K.), University of Utah School of Medicine, Salt Lake City; Cardiology and Angiology I (H.B.) and Institute for Experimental Cardiovascular Medicine (E.A.R.-Z., P.K.), Heart Center Freiburg University, and Faculty of Medicine (H.B., E.A.R.-Z., P.K.), University of Freiburg, Germany; Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham (A.R.W.); Department of Radiology (T.L.S., K.I.S.) and Diabetic Cardiovascular Disease Center, Cardiovascular Division (J.E.S.), Washington University School of Medicine, St. Louis, MO; Division of Cardiology, Department of Medicine, University of Colorado Anschutz Medical Center, Aurora (G.C.S.); and Department of Biochemistry and Nebraska Redox Biology Center, University of Nebraska, Lincoln (O.K.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at http://circres.ahajournals.orglookup/suppl/doi:10.1161/CIRCRESAHA.117.311307/-/DC1.

Correspondence to E. Dale Abel, MBBS, DPhil, Division of Endocrinology and Metabolism, Fraternal Order of Eagles Diabetes Research Center, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, 4312 PBBB, 169 Newton Rd, Iowa City, IA 52242. E-mail DRAdmin@uiowa.edu

© 2017 American Heart Association, Inc.
ATP generation in cardiomyocytes. FAs derived from circulating triglyceride-rich lipoproteins and albumin-bound nonesterified FAs are oxidized in the mitochondrial matrix via β-oxidation, whereas pyruvate derived from glucose and lactate is oxidized by the pyruvate dehydrogenase complex, localized within the inner mitochondrial membrane. Acetyl-coenzyme A (CoA) formed from FAs and pyruvate is oxidized within the mitochondrial matrix by the citric acid cycle. Healthy adult cardiomyocytes preferentially use FAs as an energy substrate. However, energy substrate usage is flexible in normal hearts and varies to adapt to physiological or pathological stresses.

Obesity, insulin resistance, and diabetes mellitus are associated with increased fatty acid utilization and reduced glucose utilization. One proposed mechanism for cardiac dysfunction called lipotoxicity is excess intracellular lipid accumulation secondary to a mismatch between lipid supply and utilization. In this study, using a mouse model with increased myocardial lipid uptake, we demonstrate that myocardial lipid overload induces mitochondrial reactive oxygen species production, which alters the activity of DRP1 (dynamin-related protein 1) and OPA1 (optic atrophy 1), leading to fragmentation of the mitochondrial network characterized by a narrow tubular morphology. Thus, dysregulation of mitochondrial dynamics after lipid overload may represent an important mechanism contributing to mitochondrial and contractile dysfunction in lipotoxic cardiomyopathies.
to the long-chain FA palmitate. Mechanistically, these changes in mitochondrial morphology reflected changes in mitochondrial dynamics that were mediated via ROS-induced post-translational modification of mitochondrial proteins, AKAP121 (A-kinase anchor protein 121), DRP1 (dynamin-related protein 1), and OPA1 (optic atrophy 1). Thus, lipid overload leads to dysregulation of mitochondrial dynamics, which may impair mitochondrial bioenergetics in diabetic cardiomyopathy.

Methods
The authors declare that all supporting data are available in the article (and its online supplementary files in the Online Data Supplement).

Detailed methods can be found in the Online Data Supplement.

Results
Increased FA Uptake Elevates Myocardial Ceramide and Diacylglycerol Content and Induces Cardiac Hypertrophy and Modest Systolic Dysfunction
To investigate the consequences of persistent lipid overload in the heart, we examined mice with low-level overexpression of ACSL1 driven by the cardiomyocyte-specific α-MHC (myosin heavy chain) promoter (Acsl1 transgenic [ACStg] mice).

Because our goal was to examine mitochondrial adaptations before the onset of lipotoxic cardiomyopathy, we studied the line with the lowest level of ACSL1 overexpression, which does not develop overt heart failure until animals are 4 months old. Transgene expression was examined through Western blot and ACSL1 protein was undetectable by Western blot in both wild-type (WT) and ACStg mice at birth (P0 [postnatal day zero]) despite ACSL1 mRNA expression being 13-fold greater in ACStg neonates compared with WT neonates (Figure 1A and 1B). Increased ACSL1 protein in ACStg hearts was observed after P7 (Figure 1A). Palmitate biodistribution measured by in vivo positron emission tomography at 12 weeks of age was increased 2-fold, indicating increased cardiac FA uptake in ACStg hearts (Figure 1C). At this time point, there was no increase of myocardial triglyceride content in ACStg hearts under random fed conditions (Figure 1D).

Myocardial ceramide and diacylglycerol content were significantly increased in ACStg hearts (Figure 1E and 1F). Consistent with the previous report, this moderate increase of lipid uptake induced a modest increase in ventricular weight/body weight ratio and ventricular wall thickness (interventricular septum) on the basis of cardiomyocyte hypertrophy in ACStg hearts (Figure 1G through 1I; Online Figure I). Although there was no statistically discernable difference in left ventricular end-diastolic volume and left ventricular ejection fraction measured by echocardiography (Figure 1J and 1K), cardiac catheterization revealed a significant reduction in arterial blood pressure in ACStg mice accompanied by decreased max dP/dt and min dP/dt by the age of 24 weeks (Figure 1L through 1N).

Furthermore, B-type natriuretic peptide expression and Acta1 (α-skeletal muscle actin) expression were modestly increased in ACStg hearts by 2-fold (B-type natriuretic peptide) and 1.3-fold (Acta1) at the age of 12 weeks (Figure 1O). Taken together, a 2-fold increase of lipid uptake induces modest cardiac hypertrophy with preserved systolic function at the age of 12 weeks but impairs systolic function at the age of 24 weeks.

Increased Respiration and ROS Production in Isolated Mitochondria From ACStg Hearts
In ACStg mice, oxygen consumption rates (OCRs) and ATP synthesis rates in isolated mitochondria incubated with palmitoyl-carnitine as a substrate were increased at 12 weeks of age, but were unchanged at 24 weeks of age relative to WT controls (Figure 2A and 2B). In contrast, OCRs were not impaired with pyruvate or glutamate as substrates at 12 and 24 weeks of age, respectively (Figure 2C and 2D). In addition, in-gel activities (in blue-native PAGE) of oxidative phosphorylation complexes I and IV were unchanged, but complex V activity was increased at 12 weeks of age in ACStg hearts (Figure 2E through 2G).

Mitochondrial superoxide production (measured as H2O2 release from mitochondria) was increased 2-fold in ACStg heart mitochondria exposed to succinate or palmitoyl-carnitine as a substrate and was completely inhibited by the addition of rotenone (Figure 2H). Increased ROS was not observed when glutamate was used as a substrate (Online Figure II A), suggesting that ROS production derives from reducing equivalents that are oxidized by complex II. Oxidation of 2,7′-dichlorofluorescein diacetate was increased in whole-cell extracts of ACStg hearts (Figure 2I). Moreover, increased mitochondrial 4HNE (4-hydroxy-2-noneal) adducts in ACStg mitochondrial proteins, a highly toxic aldehyde byproduct of lipid peroxidation caused by ROS production, also support increased mitochondrial ROS production (Figure 2J). However, activity of mitochondrial aconitase was not reduced, suggesting that there was no increase in oxidative stress in the mitochondrial matrix of ACStg hearts, potentially the result of increased superoxide dismutase 2 (SOD2) content (Figure 2K; Online Figure II B). Thus, short-term low-level lipid overload does not initially impair mitochondrial oxidative capacity in the murine heart despite increasing ROS generation.

Postnatal Increase in Mitochondrial Cross-Sectional Area Is Attenuated in Lipid-Overloaded Cardiomyocytes
We next investigated mitochondrial morphology. During the early postnatal period, cardiomyocytes dynamically change their structure and function adapting to the increased workload and oxygen consumption that parallels the switch of substrate utilization from glycolysis to mitochondrial FA oxidation. Mitochondrial morphology also changes, and volume density increases as they become larger and more ovoid in shape. Two-dimensional electron microscopy revealed that mitochondrial dimension in WT hearts increased 2-fold within the first 3 weeks after birth before plateauing (Figure 3A; Online Figure III A). This postnatal mitochondrial enlargement was absent in ACStg mice (Figure 3A; Online Figure III A). In contrast, mitochondrial volume density was significantly increased in ACStg hearts (Figure 3A and 3B; Online Figure III B and III C), based on the appearance of accumulated small mitochondria. To evaluate mitochondrial morphology more precisely, we performed 3D transmission electron microscopic tomography at the age of 8 weeks. Three-dimensional mitochondrial images revealed the presence of narrow but elongated mitochondria whose tortuous...
shapes crossed individual sectioning planes more than once (Figure 3B and 3C). This is associated with an increase in number of cytosol–mitochondrial membrane transitions on any projection between the M-lines of neighboring myofilament bundles (Figure 3D). The short-axis diameter of mitochondria was significantly reduced in ACStg hearts by 60%
Figure 2. Mitochondrial respiratory function and reactive oxygen species (ROS) production in isolated mitochondria from Acsl1 (long-chain acyl-CoA synthetase 1) transgenic mice (ACStg) hearts. A, B, Maximal ADP-stimulated mitochondrial oxygen consumption (A) and ATP synthesis rates (B) in mitochondria isolated from 12- and 24-wk-old WT and ACStg hearts using palmitoyl-carnitine as a substrate; n=4 to 5. *P<0.05 vs wild type (WT). C, D, Maximal ADP-stimulated mitochondrial oxygen consumption in saponin-permeabilized cardiac fibers of 12- and 24-wk-old WT and ACStg hearts using pyruvate (C) or glutamate (D) as a substrate; n=6. E–G, Electrophoretic separation of oxidative phosphorylation (OXPHOS) complexes by blue-native PAGE (E), representative images of in-gel activities of complexes I, IV, and V (F), and quantification of OXPHOS complex activities (G), measured in 12-wk-old WT and ACStg hearts; n=4. *P<0.05 vs WT. H, H$_2$O$_2$ production with succinate as a substrate or with palmitoyl-carnitine as a substrate in mitochondria isolated from 12- or 24-wk-old WT and ACStg hearts, in the absence of rotenone (Rot); n=3 at 12 wk, n=6 at 24 wk. *P<0.05 vs WT. I, Oxidation of 2′,7′-dichlorofluorescein-diacetate (DCFDA) in whole-tissue extracts of 24-wk-old WT and ACStg hearts; n=5 to 6. *P<0.05 vs WT. J, Western blot for 4HNE (4-hydroxy-2-nonenal) protein adducts in mitochondrial protein isolated from WT and ACStg hearts. Numbers beneath each lane represent densitometry of 4HNE immunoreactivity of all bands in that lane. K, Aconitase activity measured in mitochondria isolated from 12- or 24-wk-old WT and ACStg hearts; n=3 at 12 wk, n=6 at 24 wk. All data are mean±SEM.
Figure 3. Mitochondrial fragmentation in Acs11 (long-chain acyl-CoA synthetase 1) transgenic mice (ACStg) hearts. A, Postnatal mitochondrial enlargement was attenuated in ACStg hearts. Stereologic quantification of mitochondrial minimum diameter and volume density was performed at the ages as indicated in 2-dimensional (2D) electron micrographs (EM) presented in Online Figure IIIA; n=3 to 4. *P<0.05, **P<0.01, ***P<0.001. B, Representative EM of longitudinal sections of wild type (WT) and ACStg cardiomyocytes (Continued)
protein kinase A and its ability to phosphorylate DRP1 is mediated by ubiquitin/proteasome-mediated degradation of AKAP121 (A-kinase anchor protein 121), which is a mitochondrial outer membrane scaffold protein.\textsuperscript{16—20} We therefore examined whether AKAP121 protein content might be decreased in ACStg hearts. In 12-week-old ACStg mouse hearts, Western blotting revealed that DRP1 phosphorylation at S637 was significantly reduced and phosphorylation at S616 increased (Figure 4A through 4C). In addition, AKAP121 expression was also significantly decreased in ACStg hearts relative to WT (Figure 4A and 4D), which was not related to any difference in AKAP121 mRNA expression (Figure 4E). Therefore, we tested whether post-translational regulation by the ubiquitin/proteasome system might increase AKAP121 degradation in the face of lipid overload. WT or ACStg mice were injected intraperitoneally with MG132 (carbobenzoxy-Leu-Leu-leucinal) or DMSO and whole-heart homogenates were subjected to Western blot analysis. The proteasome inhibitor MG132 blocked the reduction of AKAP121 protein content in ACStg mice, suggesting that AKAP121 is degraded by the ubiquitin/proteasome system pathway in response to lipid overload (Figure 4F). AKAP121 is a target of the ubiquitin ligase Siah2 (seven in absentia homolog 2) in the context of hypoxia.\textsuperscript{20} However, Siah2 mRNA expression was not increased in ACStg hearts (Figure 4G). Taken together, lipid overload altered DRP1 phosphorylation by enhancing AKAP121 degradation which may be independent of the hypoxia-stimulated pathway.

We also examined whether the mitochondrial fusion protein, OPA1, could be affected by lipid overload. There are at least 8 mRNA variants of OPA1 as a result of alternative splicing.\textsuperscript{21} Variants 1 and 7 are dominant variants in mammalian cells. In response to mitochondrial depolarization, OPA1 also undergoes proteolytic cleavage which impairs its ability to induce mitochondrial fusion.\textsuperscript{22} Western blot analysis of heart mitochondrial protein showed 5 bands of OPA1, 2 fusion-competent long isoforms and the 3 fusion-incompetent short isoforms generated by the proteolytic processing of the 2 long isoforms (Figure 4H). Consistent with enhanced mitochondrial network fragmentation in ACStg hearts, the ratio of short form to long form was increased by 1.7-fold in ACStg hearts (Figure 4H and 4I). Thus, these data support the hypothesis that mitochondrial dynamics shifts toward enhanced mitochondrial fission by at least 2 distinct mechanisms.
Effect of Long-Chain FA, Palmitate, and Oleate on Mitochondrial Respiration and ROS Production in Rat Neonatal Cardiomyocytes

To more directly determine the mechanistic basis for the mitochondrial adaptation to lipid overload, we treated cultured NRVCs with free FAs. Because saturated FAs are the main mediators of lipotoxicity, we treated NRVCs with BSA (bovine serum albumin)-conjugated palmitic acid (C16:0) to mimic lipid overload in vivo and compared them with cells treated with the mono-unsaturated FA, oleic acid (C18:1).
Oxygen consumption of lipid-treated NRVCs was determined with the Seahorse XF24 flux analyzer. Although short exposure to palmitate (<4 hours) increased mitochondrial OCRs in NRVCs, continued exposure (>8 hours) reduced OCR (Figure 5A and 5B; Online Figure VA and VB). In contrast, oleate treatment did not alter mitochondrial OCR (Figure 5A). To confirm that this increase of OCR is coupled to mitochondrial oxidative phosphorylation and ATP synthesis, we examined...

Figure 5. Mitochondrial metabolism and reactive oxygen species (ROS) production in rat neonatal cardiomyocytes after increasing duration of free fatty acids exposure. A, Basal oxygen consumption rate (OCR; assayed by the Seahorse XF24 system) in neonatal rat ventricular cardiomyocytes (NRVCs) incubated with BSA (bovine serum albumin) alone, palmitate-BSA (500 μmol/L) or oleate-BSA (500 μmol/L). **P<0.01. B, Time course analysis of OCR of NRVCs treated with palmitate-BSA. Increased basal OCR after short-term exposure to palmitate (3 h) and reduced basal OCR after long-term exposure (15 h) of palmitate. **P<0.01 (Online Figure V). C, Time course analysis of ATP content in NRVCs after palmitate or oleate treatment. **P<0.01 vs BSA, #P<0.05. D, Time course analysis of mitochondrial polarization (membrane potential) after palmitate or oleate treatment. Cardiomyocytes were stained with TMRM (tetramethylrhodamine, methyl ester) and Hoechst at indicated times and fluorescence intensity was assayed with a plate reader. The ratio of TMRM/Hoechst fluorescence is shown. n=4, *P<0.05 vs BSA, #P<0.05. E, Western blot for 4HNE (4-hydroxy-2-nonenal) protein adducts in NRVCs after palmitate or oleate treatment. F, G, CellROX green staining in NRVCs after palmitate treatment. CellROX Green is a DNA dye, and on oxidation, it binds to DNA. Representative confocal image (F) and fluorescence intensity were quantified (G). **P<0.01 vs BSA. All data are means±SEM. Scale bars indicate 20 μm.
Figure 6. DRP1 (dynamin-related protein 1) mediates mitochondrial fission after lipid overload. A. Rat neonatal cardiomyocytes were stimulated with growth medium with or without 500 μmol/L palmitate or oleate. Twelve hours after stimulation, cells were fixed with 4% paraformaldehyde and immunostained with α-actinin (green), Tom20 (translocase of outer membrane 20kDa, red), and DAPI (blue). Scale bars indicate 20 μm. B. Quantification of mitochondrial fragmentation presented in A. More than 100 cells were counted to determine the percentage (%) of cells with fragmented mitochondria. n=3, **P<0.01. C. Increased phosphorylation of DRP1 at Ser616 after palmitate treatment. Neonatal rat ventricular cardiomyocytes (NRVCs) were treated in growth medium with 500 μmol/L palmitate, and cell lysates were harvested at the indicated times in hour. D. NRVCs were treated in growth medium with or without 500 μmol/L palmitate and subjected to immunohistochemistry for DRP1 (green) and Tom20 (red). Note that DRP1 is colocalized with Tom20 after palmitate treatment. Scale bars indicate 20 μm. E. NRVCs were infected with AdGFP (adenovirus encoding GFP) or Ad DRP1K38E and were treated in growth medium with 500 μmol/L palmitate. NRVCs were subjected to immunohistochemistry for α-actinin (green) and Tom20 (red; Online Figure VIIA). Scale bars indicate 20 μm. F. Representative electron micrographs of longitudinal heart sections obtained from wild type (WT), DRP1+/−, Acsl1 (long-chain acyl-CoA synthetase 1) transgenic mice (ACStg) and ACStg×DRP1+/−. DRP1 knockdown partially restored mitochondrial morphology in ACStg hearts (Online Figure VIIB through VIIE). pERK indicates phosphorylated extracellular-signal regulated kinase; and pJNK, phosphorylated c-jun N-terminal kinase.
time course changes of mitochondrial membrane potential and ATP content after palmitate treatment. Consistent with OCR, palmitate treatment initially increased mitochondrial polarization and ATP content, but these parameters declined after continued incubation for >12 hours (Figure 5C and 5D).

We also examined ROS production of cardiomyocytes after palmitate or oleate treatment. As expected, Western blotting of 4HNE revealed that ROS production was increased after palmitate treatment (Figure 5E). CellROX Green (ROS probe) also accumulated in the nucleus after 12 hours of palmitate treatment (Figure 5F and 5G). In contrast, oleate treatment did not increase 4HNE accumulation (Figure 5E). Taken together, palmitate supplementation initially accelerates mitochondrial respiration that is coupled to ATP synthesis. However, continued exposure to palmitate induces mitochondrial respiratory failure, potentially on the basis of ROS-induced injury.

Figure 7. Mitochondrial fusion in L6 myoblasts was impaired by palmitate exposure. A, L6 myoblasts transfected with mitoRFP (mitochondrial-red fluorescent protein) were incubated in growth medium with or without 500 μmol/L palmitate for 5 h, and representative images are shown. B, Cells were evaluated to quantify tubular or fragmented mitochondrial networks, and cells with fragmented mitochondria were expressed as percentage of all viewed cells, n=4 and 80 cells counted per group. **P<0.01 vs BSA. All data are mean±SEM. C, L6 myoblasts were transfected either with mitoRFP or mitoGFP (mitochondrial-green fluorescent protein), coplated (25 000 cells each) on cover slips for 24 h, and then cell fusion was induced with polyethylene glycol (PEG). Cells were fused for 8 h in regular growth medium with no palmitate (top row), 8 h in growth medium with 500 μmol/L palmitate, after 3 h preincubation with 500 μmol/L palmitate before PEG fusion (middle row), or 8 h in the regular growth medium with no palmitate, after 3 h preincubation with 500 μmol/L palmitate before PEG fusion (bottom row).
Figure 8. Mitochondrial superoxide dismutase (SOD2) overexpression partially rescued abnormal mitochondrial dynamics in Acsl1 (long-chain acyl-CoA synthetase 1) transgenic mice (ACStg) mice. A, B, Enhanced reactive oxygen species (ROS) production in ACStg hearts was rescued by SOD2 overexpression. H$_2$O$_2$ production was determined in isolated mitochondria from 12-wk-old wild type (WT), SOD2tg, ACStg, and SOD2×ACS double tg hearts in the absence or presence of rotenone (A). Mitochondrial fractions were prepared from 12-wk-old WT, SOD2tg, ACStg, and SOD2×ACS double transgenic hearts and subjected to Western blot for 4HNE (4-hydroxy-2-nonenal) and quantified by densitometry (B), n=3 to 4 in each group. **P<0.01 (Online Figure VIIIA; C) SOD2 overexpression partially rescued fragmented mitochondria in ACStg hearts. Representative electron micrographs from WT, SOD2tg. (Continued)
Altered Mitochondrial Dynamics in Response to Lipid Overload Is Mediated in Part by Post-Translational Modification of DRP1

We next determined whether mitochondrial dynamics is modulated by FAs in NRVCs. To visualize the mitochondrial network, cardiomyocytes were stained with mitotracker in live cells or immunostained with an anti-Tom20 (translocase of outer membrane 20 kDa) antibody in fixed cells. The normal mitochondrial network in NRVCs appeared as tubular mitochondria (Figure 6A; Online Figure VIA). Palmitate supplementation changed mitochondrial morphology from a tubular to small rounded shape (Figure 6A and 6B; Online Figure VIA). Interestingly, these morphological changes were not observed in oleate-treated cardiomyocytes (Figure 6A and 6B). We examined DRP1 phosphorylation profiles in NRVCs after palmitate treatment. Similar to P0 hearts (Figure 3G and 3H), we detected no phosphorylation at S637 in isolated NRVCs throughout the time course. Instead, palmitate treatment increased DRP1 phosphorylation at S616 in NRVCs (Figure 6C). Immunohistochemistry revealed DRP1 translocation from the cytosol to mitochondria in response to palmitate treatment (Figure 6D). To validate that DRP1 mediates mitochondrial network fragmentation in lipid overload, we examined the effect of DRP1K38E, a dominant negative mutant of DRP1.25 DRP1K38E overexpression prevented palmitate-induced mitochondrial network fragmentation in NRVCs (Figure 6E; Online Figure VIIIA). Furthermore, the reduced mitochondrial cross-sectional area observed was partially reversed in ACStg mice that lacked 1 DRP1 allele in cardiomyocytes (ACStg DRP1+/−; Figure 6F; Online Figure VIIIB through VIIIE). Taken together, palmitate exposure shifts mitochondrial dynamics toward fission and away from fusion, in part via a DRP1-mediated mechanism.

Mitochondrial morphology reflects the balance of mitochondrial fusion and fission. To directly evaluate if palmitate exposure inhibits mitochondrial fusion leading to mitochondrial network fragmentation, we cocultured L6 myoblasts expressing either mitoGFP (mitochondrial-green fluorescent protein) or mitoRFP (mitochondrial-red fluorescent protein) and induced cell fusion with polyethylene glycol. L6 myoblasts displayed mitochondrial network fragmentation which was evident after 3 hours of palmitate supplementation (Figure 7A and 7B). Mitochondrial network fragmentation was further augmented when expression of ACSL1 was increased after retroviral transduction (Online Figure VIB). After the induction of cell fusion with polyethylene glycol, fusion of GFP and RFP-labeled mitochondria was evident in cells treated with control media when imaged 8 hours after polyethylene glycol–induced cell fusion (Figure 7C, top). In contrast, mitochondrial fusion was not observed in the cells incubated with palmitate-supplemented medium (Figure 7C, middle). To determine if palmitate pre-exposure permanently impacted mitochondrial fusion in L6 myoblasts, cells were pretreated with palmitate-supplemented medium for 3 hours that disrupted the mitochondrial network and then the medium was replaced with normal growth medium. Mitochondria regained their tubular structure after removal of palmitate, and fusion of GFP and RFP mitochondrial populations was observed, suggesting that palmitate-induced defects in mitochondrial fusion are reversible (Figure 7C, bottom).

Contribution of Mitochondrial ROS Generation to FA-Mediated Mitochondrial Fragmentation

In light of our observations of increased lipid-mediated ROS production in vivo and in vitro that correlated with mitochondrial network fragmentation, we tested the hypothesis that elevated mitochondrial ROS production may initiate downstream signaling events to alter mitochondrial dynamics. Because mitochondrial SOD2 mediates superoxide detoxification at a diffusion-limiting rate in the mitochondrial matrix,26 we examined whether SOD2 overexpression could rescue altered mitochondrial morphology in ACStg hearts. We generated a cardiac-specific double transgenic mouse harboring SOD2 and ACSL1 transgenes (ACs×SOD2 mice). As expected, enhanced superoxide production and increased 4HNE adducts observed in ACStg mitochondria were almost completely rescued by the overexpression of SOD2 (Figure 8A and 8B; Online Figure VIIIIB). Mitochondrial cross-sectional area and apparent number were normalized in ACStg hearts by SOD2 overexpression (Figure 8C; Online Figure VIIIB). Surprisingly, SOD2 overexpressing cardiomyocytes with reduced ROS production demonstrated a 4-fold increase in mitochondrial cross-sectional area estimated from 2D images, suggesting that mitochondrial ROS levels are inversely correlated with mitochondrial dimensions (Figure 8A and 8C).

Finally, we determined whether altered DRP1 phosphorylation or changes in OPA1 processing in ACStg mice were prevented by SOD2 overexpression. Western blot analysis showed that protein expression of AKAP121 and DRP1 phosphorylation at S637 was increased and DRP1 phosphorylation at S616 was reduced by SOD2 overexpression (Figure 8D and 8E). In addition, total levels of DRP1 normalized to voltage-dependent anion channel, which were increased in ACStg hearts, were normalized by SOD2 overexpression (Figure 8F and 8H). Overexpression of ACSL1 enhanced OPA1 proteolysis to specifically increase short isoform-d formation (Figure 8F and 8G). Relative to ACStg, in SOD2/ACS double transgenic mice, there was a reduction in isoform-d and an increase in isoform-e (Figure 8F and 8G). In vitro, MnTBAP (manganese [III] tetra-kis [4-benzoic acid] porphyrin chloride), the SOD mimetic and peroxynitrite scavenger, partially normalized palmitate-induced mitochondrial network fragmentation in NRVCs (Figure 8I and 8J).
Alter ed Mitochondrial Dynamics in Lipotoxic Hearts

Discussion
Recent studies have suggested that impaired mitochondrial energetics may contribute to the increased risk of heart failure in type-2 diabetes mellitus. The pathophysiology of mitochondrial dysfunction in diabetes mellitus is complex and may include altered insulin signaling, glucotoxicity, and lipotoxicity. Prior work from our group suggested that impaired myocardial insulin signaling could precipitate mitochondrial dysfunction in the heart. Given the likelihood that lipotoxicity could have distinct effects on mitochondria, the present study was designed to model increased myocardial lipid accumulation to a level that was similar in magnitude to that reported in animal models of obesity and diabetes mellitus. Therefore, we used mice with cardiomyocyte-specific, low-level overexpression of ACSL1, which gradually developed myocardial dysfunction and recapitulated key observations in neonatal cardiomyocytes that were incubated with palmitate. First, we observed that lipid overload initially enhances mitochondrial respiration coupled to ATP synthesis. However, prolonged lipid overload enhanced mitochondrial ROS generation, which is followed by reduced mitochondrial respiration and ATP synthesis. Second, we observed differential metabolic fates of palmitate and oleate in NRVCs. Our data indicate that increased mitochondrial respiration after palmitate supplementation results in accumulation of mitochondrial ROS and impaired mitochondrial energetics. Conversely, oleate supplementation did not increase mitochondrial respiration or ROS production and did not precipitate mitochondrial impairment. Third, mitochondrial redox status might influence mitochondrial morphology by modulating the post-translational modifications of proteins that regulate mitochondrial dynamics. Lipid overload increased AKAP121 ubiquitination, modulated DRP1 phosphorylation, and altered OPA1 processing. Future studies in models with inducible increases in myocardial lipid uptake will be needed to determine whether these mechanisms also alter mitochondrial dynamics and energetics in adult (postnatal) murine hearts. Prior studies from our laboratory showed altered mitochondrial morphology in db/db diabetic mice or type-1 diabetic Akita mice, which was accompanied by enhanced mitochondrial ROS production. A recent study also revealed that right atrial cardiomyocytes isolated from type-2 diabetic patients exhibit increased mitochondrial ROS production and impaired mitochondrial bioenergetics. Taken together, these observations suggest that the mechanism observed in the present study could be universally applicable to the pathophysiology of mitochondrial dysfunction in lipotoxic cardiomyopathy associated with obesity or diabetes mellitus.

After uptake of FAs in cardiomyocytes, ACSLs immediately catalyze FAs to Acyl-CoA esters, which enter a variety of lipid metabolic pathways such as FA oxidation, TG synthesis/storage, phospholipid metabolism, and de novo ceramide synthesis. Cardiac ACSL1 overexpression in vivo increases mitochondrial ROS production and the content of reactive lipid metabolites. We used a genetic approach to modulate mitochondrial ROS production by overexpressing ACSL1, SOD2, or both and demonstrated that mitochondrial redox status correlates with mitochondrial morphology. In addition, mitochondrial redox status is also associated with processing of mitochondrial proteins, such as OPA1 proteolysis, ubiquitin/proteasome-mediated degradation of AKAP121, and DRP1 phosphorylation at Ser637. DRP1 phosphorylation at Ser616, a target site for MAP (mitogen-activated protein) kinases and cyclin-dependent kinase 1, also correlated with mitochondrial redox status and was recapitulated by in vitro palmitate treatment of NRVCs. These data are also supported by a recent publication showing that the ROMO1 (ROS modulator 1), a mitochondrial protein, is a redox-sensitive factor that regulates mitochondrial morphology by affecting OPA1 cleavage and oligomerization.

A recently described mechanism that may also induce mitochondrial fission is hypoxia. Siah2, a RING finger ubiquitin ligase induced by hypoxia, mediates ubiquitin/proteasome degradation of AKAP121. Subsequent reduction of protein kinase A–dependent inhibitory phosphorylation of DRP1 is an important regulatory pathway for hypoxia-induced mitochondrial fission. In ACS1g hearts, we also observed substantial proteasome-mediated degradation of AKAP121, and mitochondrial redox status was correlated with AKAP121 protein levels and DRP1 phosphorylation at Ser637. However, in contrast to hypoxia, Siah2 mRNA induction was not observed in ACS1g hearts (Figure 4G), suggesting that an alternative mechanism may link the AKAP121 and DRP1 pathways in cardiac lipid overload.

Our data also reveal that substrate availability and mitochondrial dynamics play important roles in the development of mitochondria in the perinatal period. In fetal cardiomyocytes, energy substrate utilization mainly depends on lactate and glucose supplied from the maternal circulation. After birth, energy substrate utilization shifts rapidly from lactate oxidation and glycolysis to FA oxidation in mitochondria, and cardiomyocytes preferentially use FA as an energy source throughout their life. In this process, mitochondria undergo profound remodeling in terms of shape and volume, which is accompanied by an increase in the activities of enzymes required for lipid oxidation, such as long-chain acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase, carnitine palmitoyl transferase I, and ACSL1. The progressive postnatal increase in mitochondrial diameter was absent in ACS1g hearts, which showed a multitude of apparently small mitochondria occupying a larger fractional cell volume than WT. Three-dimensional electron micrograph tomographic reconstructions revealed a narrow, elongated, and tortuous morphology of mitochondria that is principally different from WT and which would be sectioned multiple times in a single plane. Our novel imaging methodology underscores the challenges in estimating mitochondrial size and number from 2D images and adds new insight into the structural adaptations of mitochondria in the heart when signaling pathways that regulate mitochondrial dynamics are perturbed.

The substantial increase of FA availability in the perinatal circulation activates PPARα (peroxisome proliferator-activated receptor-α) that drives FA oxidation in mitochondria. We show here that DRP1 phosphorylation is rapidly modulated during postnatal cardiac development. Furthermore, forced lipid uptake in the early postnatal period alters the developmental changes in DRP1 phosphorylation and prevented physiological and maturation-dependent mitochondrial fusion. An
indispensable role of DRP1 in perinatal cardiac development has been recently reported.38-40 Taken together, post-translational modifications of DRP1 after birth play an important role in normal perinatal mitochondrial maturation.

Along with the alteration of mitochondrial membrane dynamics, mitochondrial membrane lipid content was also affected in our cardiac lipid overload model. Although the detailed molecular mechanisms involved are incompletely understood, the lipid composition of cristae membrane, specifically cardiolipin and phospholipids, plays an important role in mitochondrial membrane fluidity, cristae structure, and oxidative phosphorylation.41 Consistent with an elevation of cardiac ceramide and diacylglycerol levels (Figure 1E and 1F), phospholipid composition and the side chain pattern of cardiolipin were significantly altered in ACStg mitochondrial membranes (Online Figure IXA and IXB). Interestingly, there was a significant reduction in tetranoic cardiolipin (Online Figure IXA). It is widely accepted that tetranoic cardiolipin is the fully functional cardiolipin species, and the reduction of tetranoic cardiolipin has been associated with diabetes mellitus, heart failure, ischemia–reperfusion injury, all of which are associated with impaired mitochondrial function.42,43 These changes are due in part to an important role for cardiolipin in mitochondrial supercomplex formation and cytochrome oxidase activity.44 Moreover, tetranoic cardiolipin is thought to promote more efficient proton flow, and as such the reduction observed in ACStg mice could account in part for the increased ROS generation.

We also addressed the contribution of increased ceramide content in altering mitochondrial morphology in our lipid overload model in vivo and in vitro. Using myriocin, an inhibitor for serine palmitoyltransferase, which catalyzes the first step of de novo ceramide synthesis, we determined that the inhibition of de novo ceramide synthesis exacerbated ROS production in palmitate-treated NRVCs (Online Figure XA through XE). Furthermore, in vivo inhibition of ceramide synthesis by heterozygous deletion of dihydroceramide desaturase 1, an enzyme that catalyzes de novo ceramide synthesis, failed to reverse the altered mitochondrial network (Online Figure XF). These data indicate that increased ROS resulting from mitochondrial lipid oxidation likely represents a primary mechanism responsible for augmenting mitochondrial fission in lipid overload and that ceramide accumulation does not contribute to this process.

In summary, we demonstrate here that increased myocardial lipid uptake impairs mitochondrial dynamics by increasing mitochondrial ROS generation, which modulates post-translational modification of OPA1 and DRP1 (Online Figure XI). We also provide evidence that mitochondrial dynamics is involved in perinatal mitochondrial maturation and forced lipid uptake interferes with that process. These findings add a new dimension to understanding the regulation of mitochondrial morphology and physiology in the heart and its dysregulation by lipid excess.

Acknowledgments

We thank Drs Elizabeth A. Amiott and Janet M. Shaw for providing critical reagents and experimental guidance in the L6 mitochondrial fusion assays; Heather Theobald for technical help; and Dr Eric T. Weatherford and Paul Casella for the editing of the article.

References


37. Bugger H, Boudina S, Hu XX, Tuinei J, Zaha VG, Theobald HA, Yoon UJ, McQueen AP, Waymert B, Litwin SE, Abel ED. Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. *Diabetes.* 2008;57:2924–2932.


Mitochondrial Reactive Oxygen Species in Lipotoxic Hearts Induce Post-Translational Modifications of AKAP121, DRP1, and OPA1 That Promote Mitochondrial Fission


Circ Res. 2018;122:58-73; originally published online November 1, 2017;
doi: 10.1161/CIRCRESAHA.117.311307

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/122/1/58

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2017/10/31/CIRCRESAHA.117.311307.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Materials

Online Methods

Animal model
Mice with cardiomyocyte-restricted low-level overexpression of long-chain acyl-CoA synthetase 1 (ACStg) and control littermates (WT) were originally on the FVB background. We prepared a transgenic line on the C57BL6j background by backcrossing more than 8 generations. DRP1 floxed mice (Kind gift of Hiromi Sesaki, Johns Hopkins University, Baltimore MD) were used to generate cardiomyocyte-restricted heterozygous deletions of DRP1 (Mixed Background). Sod2 transgenic mice (FVB background) were a kind gift from Paul Epstein (University of Louisville, Louisville, KY) and Des1 +/- mice (C57BL6 background) were a kind gift of Scott Summers (University of Utah, Salt Lake City, UT). Animals were studied in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University Utah and the Carver College of Medicine of the University of Iowa. All mice were housed at 22°C with free access to water and food (standard chow diet) with a light cycle of 12h light and 12h dark.

In vivo cardiac function
Echocardiography or left-ventricular (LV) catheterization was performed in a subset of the mice before respiration studies as described previously. Mice were lightly anesthetized with isoflurane and imaged in the left lateral decubitus position with a linear 13-MHz probe (Vivid V echocardiograph; GE Healthcare, Tampa, FL). Cardiac dimensions and function were calculated from these digital images. Invasive LV hemodynamic measurements were performed with a temperature-calibrated 1.4-Fr micromanometer-tipped catheter (Millar Instruments, Houston, TX) inserted through the right carotid artery in mice anesthetized with choral hydrate (400 mg/kg) and analyzed as described by us previously.

In vivo palmitate uptake
**In vivo** palmitate uptake was measured by quantifying cardiac palmitate biodistribution of 1-\(^{11}\)C-palmitate using small-animal positron emission tomography (PET) as described before \(^3\).

**In vivo** MG132 administration
10mg of MG132 (EMD Millipore, Billerica, MA) was dissolved in Corn Oil (Sigma-Aldrich, St, Louis, MO) by rotating 24hrs in room temperature. MG132 (10mg/kg body weight) was administered intraperitoneally twice, prior to euthanasia (- 18h and - 6h).

**Cardiomyocyte isolation**
Primary cultures of neonatal rat ventricular cardiomyocytes (NRVCs) were prepared from the ventricles of 3-5-day-old Wistar rats as described previously \(^4\). NRVCs were plated on type I collagen coated cover glass or culture plates and incubated with DMEM supplemented with BSA or palmitate-BSA. NRVCs were also infected with adenovirus expressing either Ad-GFP or Ad-DRP1K38E at 10 MOI. The CRP-DRP1-K38E adenovirus was a gift from Dr. Ruth Slack’s laboratory, Ottawa, Canada \(^5\). After 12hr. of infection, NRVCs were incubated in DMEM or palmitate as described above.

**Mitochondrial fusion assay**
Mitochondrial fusion was investigated using the PEG fusion assay, as described before with minor modifications \(^6\). In brief, L6 myoblasts were infected with mitoGFP or mitoRFP (kind gifts from Dr. D. Chan) using retrovirus infection, and 25,000 cells expressing mitoGFP or mitoRFP were co-plated on 18mm coverslips in 6-well plates. Fusion was initiated by adding 300\(\mu\)l of 50% PEG 1500 (Roche, Indianapolis, IN) for 60s, followed by addition of 2ml DMEM and two more washing steps with DMEM. Cells were incubated with 1ml cycloheximide (30\(\mu\)g/ml) for 8 h, washed with 1X PBS, and fixed with 3.7% formaldehyde. Cells were viewed on a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) at a magnification of 630x.

**Immunofluorescence**
Adult cardiomyocytes and neonatal rat ventricular cardiomyocytes were isolated as described before. For mitochondrial staining of adult cardiomyocytes, cells were incubated in HEPES buffer (126mM NaCl, 4.4mM KCl, 1.0mM MgCl2, 1.08mM CaCl2, 11mM dextrose, 0.5mM probenecid, 24mM HEPES, pH 7.4) containing 100nM of the fluorescent potential-dependent indicator, tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR), for 30min at 37°C. Cells were then placed into a glass-bottom perfusion chamber mounted on a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany), perfused with HEPES-buffer at 37°C, and viewed. For neonatal rat ventricular cardiomyocytes grown on coverslips, cells were washed in warm PBS, fixed in 4% paraformaldehyde in PBS (15 min in room temperature), then washed in PBS, permeabilized with 0.2% TritonX in PBS (10 min in room temperature) and blocked in blocking solution (5% BSA in PBS with 0.05% Tween20, 30 min in room temperature). Antibody labeling was performed by addition of 200 μl blocking solution with primary or secondary antibodies (1–5 μg/ml) and washing with PBS containing 0.05% Tween20. Samples were mounted in 40% glycerol/PBS on glass slides and sealed with clear nail polish. Images were acquired in an Olympus FV-1000 confocal microscope or a Zeiss LSM 710 confocal microscope. The following antibodies were used for immunohistochemistry: Tom20 (Santa Cruz Biotechnology, Dallas, TX, sc-11415), α-actinin (Sigma-Aldrich, A7811) and DRP1 (Novus Biologicals, Littleton, CO, h00010059-m01).

Mitochondrial function
Mitochondrial oxygen consumption and ATP synthesis rates were measured in saponin permeabilized fibers using palmitoyl-carnitine/malate, pyruvate/malate, or glutamate/malate as substrate combinations, as described before. Mitochondria were isolated by differential centrifugation, and oxygen consumption and ATP synthesis were measured using palmitoyl-carnitine/malate, pyruvate/malate, or glutamate/malate as substrate combinations, as described before. OXPHOS complexes of isolated mitochondrial membranes were separated by blue-native gel electrophoresis, and complex activities were determined by in-gel staining assays, as described before. Mitochondrial respiration rate in NRVCs were assessed using a Seahorse XFp Extracellular Flux Analyzer with the XFp Cell Mito Stress
Test Kit (Agilent, Santa Clara, CA). In brief, NRVCs were plated at 75,000 cells/well in a 24-well Seahorse assay plate and maintained for 48 hrs with DMEM (5.5mM glucose, 10μM AraC, 5% FBS and 10% horse serum). 24 hr before the assay, media was changed to DMEM (5.5mM glucose without AraC and serum) and subjected to Seahorse analysis with DMEM (5.5mM glucose) supplemented with BSA or fatty acid-conjugated BSA.

**Mitochondrial membrane potential assay**

Mitochondrial membrane potential in NRVCs was measured by TMRM fluorescence intensity. In brief, NRVCs were plated at 50,000 cells/well in a 96-well black bottom dish. After palmitate stimulation, NRVCs were incubated with 10μM TMRM and 100μg/ml Hoechst for 30 min at 37°C. TMRM (540/580) and Hoechst (360/450) fluorescence intensity was measured by a SpectraMax3 plate reader (Molecular devices, Sunnyvale, California). The TMRM/Hoechst fluorescence ratio is used to determine the mitochondrial membrane potential.

**ATP extraction and quantification**

ATP content in NRVCs was measured by an ENLITEN ATP Assay System Bioluminescence Detection Kit for ATP Measurement (Promega, Madison, WI). In brief, NRVCs were plated in a 96-well plate at a density of 50,000 cells/well. ATP was extracted with 50μl of TCA(1%)/EDTA(4mM) buffer.

**Oxidative stress and superoxide production**

Mitochondrial H2O2 production was measured with a fluorometric assay as described before 8. Activity of aconitase was measured in isolated mitochondria using a spectrophotometric assay, and tissue ROS levels were measured by the conversion of nonfluorescent 2',7'-dichlorofluorescein-diacetate (DCFDA) to the highly fluorescent 2',7'-dichlorofluorescein (DCF), as described before 8. In NRVCs, ROS levels were measured by CellRox Green reagent in accordance with the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA).

**Quantification of mitochondrial dimension and volume density in 2D electron microscopy**
Samples for transmission electron microscopy were collected from left ventricular myocardium and fixed in 2.5% glutaraldehyde/1% paraformaldehyde, post-fixed in 2% osmium tetroxide, embedded in resin, and sectioned (80-100 nm thick), as described before. Mitochondrial volume density and dimensions of their two-dimensional (2D) cross-section were analyzed by 2D-stereology in a blinded fashion using the point counting method. Briefly, the image obtained at 3,000x magnification was overlaid with square grids. Mitochondrial volume density was calculated by a number of grids superimposed over mitochondria. Apparent mitochondria size was calculated as volume density / mitochondrial number.

**Electron microscopy and tomography**

Hearts were Langendorff-perfused with Tyrode's solution containing 139mM NaCl, 3mM KCl, 17mM NaHCO₃, 12mM D-(+)-glucose, 3mM CaCl₂ and 1mM MgCl₂ and cardioplegically arrested using a high-K⁺ (25 mM) no-Ca²⁺ modified Tyrode's solution. Cardioplegically arrested hearts were perfusion-fixed with iso-osmotic Karnovsky's fixative (2.4% sodium cacodylate, 0.75% paraformaldehyde, 0.75% glutaraldehyde; 300 mOsm). Tissue fragments were excised from the left ventricle and washed with 0.1 M sodium cacodylate, post-fixed in 1% OsO₄ for 1 h, dehydrated in graded acetone, and embedded in Epon-Araldite resin. Thin (80 nm) and semi-thick (280 nm) sections were placed on formvar-coated slot-grids, post stained with 2% aqueous uranyl acetate and Reynold's lead citrate. Colloidal gold particles (15 nm) were added to both surfaces of the semi-thick sections to serve as fiducial markers for tilt-series alignment. Preparations were imaged at the EMBL Heidelberg Electron Microscopy Core Facility using a Tecnai F30 electron microscope operating at 300 kV and Phillips CM120 BioTwin electron microscope operating at 120 kV. Images were captured on a 4K Eagle camera and SIS 1K KeenView, respectively.

For tomography, the SerialEM software package was used. The specimen holder was tilted from +60° to -60° at 1° intervals, followed by 90° rotation in the X-Y plane and a second round of acquisition (dual axis tilt). The images from each tilt-series were aligned by fiducial marker tracking and back-projected to generate two single full-thickness reconstructed volumes (tomograms), which were then combined to
generate a single high-resolution 3D reconstruction of the original partial cell volume. Isotropic voxel size was 1.25 nm. All tomograms were processed and analysed using IMOD software, which was also used to generate 3D models of relevant structures of interest\textsuperscript{10}.

**Immunoblot analysis**
Mitochondrial and cytosolic fractions were generated by homogenizing freshly excised hearts in homogenization buffer (20mM HEPES, 140mM KCl, 10mM EDTA, 5mM MgCl\textsubscript{2}, pH 7.4) with a Dounce tissue homogenizer, centrifuging the homogenate at 800 x g for 10min, and centrifuging the resulting supernatant at 8,000 x g for 10min. The supernatant is the cytosolic fraction. The pellet was washed by centrifugation at 10,000 x g and represents the mitochondrial fraction. Whole-cell extracts and mitochondrial membranes were prepared as described before\textsuperscript{11}. Samples were loaded on SDS-PAGE, transferred to nitrocellulose or PVDF membranes, and incubated with specific antibodies. Bands were visualized using horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (GE Healthcare, Piscataway, NJ), or fluorophore-conjugated secondary antibodies and the Odyssey fluorescence detection system (Li-Cor Biosciences, Alpharetta, GA). The following antibodies were used for immunoblotting: ACSL1 (Cell Signaling Technology, Boston, MA, #4047), phospho-ERK1/2 (Cell Signaling Technology, #9101), phospho-SAPK/JNK (Cell Signaling Technology, #9251), DRP1 pS616 (Cell Signaling Technology, #3455), DRP1 pS637 (Cell Signaling Technology, #6319), DRP1 (Novus Biologicals, h00010059-m01), 4-Hydroxyneonal (4HNE) (Abcam, Cambridge, MA, ab46545), Mitofusin 1 (Abcam, ab57602), OPA1 (BD Biosciences, San Jose, CA, #612606), Mitofusin 2 (Sigma-Aldrich, M6319), Fis1 (Enzo Life Sciences, Farmingdale, NY, ALX-210-1037-0100), MnSOD (Enzo Life Sciences, ADI-SOD-110-D).

**Cardiac mitochondrial lipid analysis**
For mass spectrometry sample preparation, tissue pieces were homogenized with a glass on glass homogenizer in PBS and lipids extracted according to a modified Bligh and Dyer method\textsuperscript{12,13} using 1,000 nmol tetramyristal cardiolipin as an internal
standard (Avanti Polar Lipids). Cardiolipin was quantified using our previously published methods\textsuperscript{12} using liquid chromatography coupled to electrospray ionization mass spectrometry in an API 4000 mass spectrometer.

**Statistical analysis**
Results are presented as means ± SEM. Data were analyzed using unpaired student T-tests. If more than two groups were compared, 1-way ANOVA was performed, and significance was assessed using Fisher’s protected least significance difference test. For T-tests and ANOVA, the Graphpad and Statplus software package was used (SAS Institute, Cary, NC). For all statistical analyses, significant difference was accepted when $P < 0.05$.


8. Bugger H, Boudina S, Hu XX, Tuinei J, Zaha VG, Theobald HA, Yun UJ, McQueen AP, Wayment B, Litwin SE and Abel ED. Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. *Diabetes.* 2008;57:2924-32.


Online Figure I

Representative images of wheat germ agglutinin staining of transverse heart sections obtained from 24-week-old ACStg mice and age-matched controls. Myocyte cross-sectional area were calculated from these images and presented in Figure 1H. Scale bars indicate 20µm.
(A) H$_2$O$_2$ production with glutamate or succinate as a substrate in mitochondria isolated from 12-week-old WT and ACStg hearts. n=3, *;P<0.05 vs WT

(B) Western blot of mitochondrial protein isolated from WT and ACStg hearts. Mitochondrial content of superoxide dismutase (SOD2 or MnSOD) and VDAC protein in 12-week-old WT and ACStg mice.
(A) Representative electron micrographs of longitudinal (24 hours, 3 weeks) or transverse (vertical) sections of WT and ACStg hearts. Scale bars indicate 500nm.

(B) Representative confocal images of cardiomyocytes isolated from 12-week-old WT and ACStg hearts, loaded with TMRE for 30 min. Scale bars indicate 20μm.

(C) Mitochondrial volume density was measured in 19 electron tomograms from 3 hearts per genotype; ** P < 0.01.
(A) Quantification of western blot presented in Figure 3G. Postnatal change of DRP1 phosphorylation in Wild-type whole heart homogenates.

(B) Quantification of western blot presented in Figure 3H. Postnatal change of DRP1 phosphorylation was compared between Wild-type and ACS transgenic mouse hearts.
(A) NRVCs were pretreated with BSA or palmitate (500μM) for 3h or 15h as indicated and oxygen consumption rate (OCR) was measured with the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Oligomycin (4μM), FCCP (1μM) + Antimycin A (1μM) were sequentially added (indicated by lines). Short term palmitate treatment (3h) increased basal and maximum OCR. However, long term palmitate treatment (15h) decreased basal and maximum OCR.

(B) NRVCs were pre-treated with BSA or palmitate (500μM) for 1h, 4h or 8h as indicated and OCR was measured with the XF24 Extracellular Flux Analyzer. Basal OCR was increased by palmitate treatment. Reduced maximum OCR was observed after 8h of palmitate treatment.
Online Figure VI

(A) Rat neonatal cardiomyocytes were incubated with culture medium supplemented with BSA or BSA conjugated palmitate (500μM) for 12-hours. Mitochondria were stained with Mitotracker green according to the manufacturer’s protocol. **; P < 0.01 vs. WT, Scale bars indicate 20μm.

(B) A representative Western blot for FLAG and ACSL1. I_{EV} - lysate of L6 cells transfected with empty virus; I_{ACSL1} - lysate of L6 cells transfected with ACSL1 virus. Cells infected with empty virus or ACSL1 virus were incubated in high-glucose DMEM + 10% FBS + 1% penicillin/streptomycin in the absence or presence of 500μM palmitate for 5 hours. Cells were evaluated to contain a tubular or fragmented mitochondrial network, and cells with fragmented mitochondria were expressed as percentage of all viewed cells n=4, * p<0.05 vs. Empty virus.
(A) Quantification of confocal images shown in Figure 6E. Mitochondrial aspect ratio was quantified by ImageJ as shown previously (Koopman et al., 2008).

(B-C) Western blot images of DRP1 protein content in whole heart lysates of Wild-type (WT), DRP1+/− (DRP1ht), ACStg and compound mutant mice (B) and densitometric quantification of the immunoblot (C).

(D-E) Stereologic quantification of mitochondria size (D) and volume density (E) in WT, DRP1ht, ACStg and ACStg x
Online Figure VIII

(A)

Mitochondrial fractions were prepared from 12-week-old WT, SOD2tg, ACStg and SOD2 x ACS double transgenic hearts and subjected to western blot for 4HNE

(B) Quantification of mitochondrial size obtained by blind counting of 2 equivalent sections from each of three separate hearts in each group depicted in Figure 6F ** P <0.01.
Online Figure IX

(A) Cardiolipin composition in mitochondrial membranes isolated from 12 week-old WT and ACStg hearts measured by mass spectrometry (LC-MS/MS); n=6.

(B) Content of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) in mitochondrial membranes of 12 week-old WT and ACStg hearts measured by thin-layer chromatography; n=6.

Data in (A) and (B) are expressed as fold change relative to WT, which was set to 1 (dashed line). * p<0.05 vs. WT
De novo ceramide synthesis inhibition does not rescue mitochondrial fragmentation after lipid overload

(A) Lipids were extracted from NRVCs treated at the indicated time points after palmitate treatment and separated by thin layer chromatography (TLC). Lipids were visualized with sulfuric acid spray. Lane 1 is C16-ceramide control and arrow indicates the position of ceramide. (B) Lipid was extracted from NRVCs by chloroform/methanol (2:1) and separated by TLC. Myriocin inhibited the increase in ceramide level after palmitate treatment. (C) ROS production in NRVCs were visualised with CellROX green reagent (Thermo Fisher Scientific). ROS production was significantly increased after palmitate treatment. The blockade of ceramide de novo synthesis by myriocin enhanced ROS production. Scale bars indicate \( \mu \text{m} \). (D) The synthesis of 4-HNE protein adducts were enhanced in myriocin treated groups. (E) Mitochondria were immunostained with an anti-Tom20 antibody. Representative images of NRVCs incubated in culture medium supplemented with BSA or 500\( \mu \text{M} \) palmitate in the presence or absence of 50\( \mu \text{M} \) myriocin, and quantification of fragmented mitochondria. Scale bars indicate 2\( \mu \text{m} \). (F) Representative electron micrographs of longitudinal sections from 12-week-old wildtype (WT), ACStg, Des1\(^{1/-} \) and ACStg x Des1\(^{1/-} \) hearts. Inhibition of the ceramide de novo synthesis pathway could not rescue mitochondrial fragmentation and proliferation in ACSStg hearts. Scale bars indicate 2\( \mu \text{m} \).
Online Figure XI
Myocardial lipid overload, by saturated fatty acids, transiently increases mitochondrial respiration and ATP generation. Prolonged exposure to lipids increases mitochondrial ROS that modifies Drp1 and OPA1 to impair mitochondrial dynamics and function in the heart, contributing to cardiac hypertrophy and dysfunction.