Tafazzin deficiency impairs CoA-dependent oxidative metabolism in cardiac mitochondria

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Barth syndrome is a mitochondrial myopathy resulting from mutations in the tafazzin (TAZ) gene encoding a phospholipid transacylase required for cardiolipin remodeling. Cardiolipin is a phospholipid of the inner mitochondrial membrane essential for the function of numerous mitochondrial proteins and processes. However, it is unclear how tafazzin deficiency impacts cardiac mitochondrial metabolism. To address this question while avoiding confounding effects of cardiomyopathy on mitochondrial phenotype, we utilized Taz-shRNA knockdown (TazKD) mice, which exhibit defective cardiolipin remodeling and respiratory supercomplex instability characteristic of human Barth syndrome but normal cardiac function into adulthood. Consistent with previous reports from other models, mitochondrial H2O2 emission and oxidative damage were greater in TazKD than in wild-type (WT) hearts, but there were no differences in oxidative phosphorylation coupling efficiency or membrane potential. Fatty acid and pyruvate oxidation capacities were 40–60% lower in TazKD mitochondria, but an up-regulation of glutamate oxidation supported respiration rates approximating those with pyruvate and palmitoylcarnitine in WT. Deficiencies in mitochondrial CoA and shifts in the cardiac acyl-CoA profile paralleled changes in fatty acid oxidation enzymes and acyl-CoA thioesterases, suggesting limitations of CoA availability or "trapping" in TazKD mitochondrial metabolism. Incubation of TazKD mitochondria with exogenous CoA partially rescued pyruvate and palmitoylcarnitine oxidation capacities, implicating dysregulation of CoA-dependent intermediary metabolism rather than respiratory chain defects in the bioenergetic impacts of tafazzin deficiency. These findings support links among cardiolipin abnormalities, respiratory supercomplex instability, and mitochondrial oxidant production and shed new light on the distinct metabolic consequences of tafazzin deficiency in the mammalian heart.

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was developed to overcome the embryonic lethality of full genetic ablation (24, 25). Aggressive induction of Taz shRNA during gestation markedly impairs cardiac development, leading to fetal or neonatal noncompaction cardiomyopathy and early lethality (26), consistent with the most severe BTHS cases (27). However, lower doxycycline dosing during gestation produces offspring that live well into adulthood despite ~90% Taz deficiency and characteristic abnormalities in cardiac mitochondria composition and mitochondrial morphology (24, 25). These mice exhibit exercise intolerance and growth delay consistent with human BTHS but maintain relatively normal cardiac function until ~6–7 months of age (24, 25, 28) despite impairments in cardiac mitochondrial function evident by 2–3 months of age (20–22, 28).

Understanding how tafazzin-deficient mice preserve basal cardiac function despite persistent cardioliol abnormalities and respiratory chain defects could reveal novel strategies for attenuating the development of cardiomyopathy in BTHS. These mice also provide an ideal model for resolving the impact of aberrant cardioliol remodeling on cardiac mitochondrial function without the confounding influence of cardiomyopathy. Therefore, the aim of this study was to define the cardiac mitochondrial phenotype of Taz shRNA mice through an integration of functional studies, cardiac metabolite analyses, and mitochondrial proteomic profiling, with a particular focus on elucidating the mechanism of substrate-specific respiratory impairment and identifying compensatory adaptations that manifest prior to the development of cardiac dysfunction.

Results

Taz-deficient mice maintain normal cardiac function despite cardioliol defects and mitochondrial supercomplex instability

Doxycycline-inducible Taz shRNA mice originally generated on a C57Bl/6 background at TaconicArtemis, GmbH (Köln, Germany) were obtained from Dr. Zaza Khuchua (University of Cincinnati) (24) and used to establish a colony of tafazzin-deficient (TazKD) mice at Colorado State University for these studies. As described previously (24, 28), transgenic males were mated with WT (C57Bl/6) females to generate TazKD and WT littermate mice. The offspring were genotyped by PCR analysis of tail DNA for the presence of the 381-bp Taz shRNA gene product (Fig. S1A). All mice were maintained on Purina RMH1500 chow (Gray Summit, MO) supplemented with 625 ppm doxycycline to induce Taz shRNA expression in transgenic mice (24, 25) and control for any unintended effects of doxycycline in WT mice (29). The extent of tafazzin mRNA knockdown was >95% as previously reported (24) and was confirmed at the protein level in cardiac mitochondria by immunoblot detecting the expected ~25–34-kDa tafazzin protein bands in WT, but not TazKD (Fig. 1A and Fig. S1B). The body weights of adult mice were persistently lower in TazKD (25.8 ± 0.6 g) compared with WT mice (34.0 ± 1.3 g; p < 0.01), consistent with growth delay and small stature in this model and BTHS patients (24).

Male TazKD and WT littermates were studied at 4–6 months of age, when cardioliol abnormalities are well-established but before the onset of cardiac dysfunction (21, 24, 25). To confirm this, electrospray ionization Mass spectrometry was performed on cardiac mitochondrial phospholipids, which demonstrated expected losses of total CL and LαCL with accumulation of monolysocardiolipin in TazKD versus WT mice within this age range (Fig. S1, C–E). Similarly, gas chromatographic analysis of fatty acids present in the cardiolipin fraction isolated by normal phase liquid chromatography revealed a predominant loss of linoleic acid with higher monounsaturated and saturated fatty acids in TazKD versus WT mitochondria, reflective of abundant nascent (nonremodeled) cardiolipin species (Fig. 1B). Interestingly, significant changes were also observed in the acyl composition of mitochondrial phosphatidylcholine and phosphatidylinositol (IF) mitochondria, indicating similar effects of tafazzin deficiency in these major cardiac mitochondrial subpopulations.

Loss of linoleic acid in cardiolipin is thought to disrupt its interaction with membrane proteins (10), favoring a destabilization of mitochondrial supercomplexes that has been reported in BTHS patient cells (14, 30). Consistent with previous reports (19–21), cardiac mitochondria from TazKD mice used in the present study exhibited lower levels of high-molecular-mass protein bands associated with CI–CIII–CIV supercomplexes separated by blue native–PAGE, which were confirmed to contain lower levels of CI and CIII subunits by subsequent SDS-PAGE and immunoblotting (Fig. 1D and Fig. S2). Smaller-molecular-mass CIII–CIV complexes were unaffected, as recently reported in BTHS patient–derived fibroblasts (30), whereas the succinate dehydrogenase (CII) complex was significantly lower in TazKD versus WT (Fig. 1D and Fig. S2, band 5), consistent with the findings of Dudek et al. (21). Despite these defects, heart mass (142 ± 9 in TazKD and 149 ± 4 mg in WT) and contractile function (Fig. 1E) were similar in TazKD and WT mice through at least 6 months of age, consistent with previous reports using the same doxycycline dosing regimen (24). Taken together, these studies validate the utility of this TazKD mouse model for defining the impact of impaired cardiolipin remodeling and supercomplex instability on cardiac mitochondrial function in the absence of confounding cardiomyopathy.

Substrate-specific impairment of OXPHOS capacity in Taz-deficient cardiac mitochondria

Previous studies have produced variable evidence of mitochondrial respiratory enzyme dysfunction in BTHS patient–derived cells and TazKD mice depending on the experimental approach and substrates employed (18, 21, 22), highlighting an incomplete understanding of how tafazzin deficiency impacts cardiac mitochondrial function. To address this, we performed a comprehensive analysis of cardiac mitochondrial bioenergetics using isolated organelles and permeabilized left ventricular fibers (Fig. 2). Mitochondrial protein yield and subpopulation distributions from cardiac tissue were similar between TazKD and WT (Fig. 2A). Enzymatic activities of respiratory
Figure 1. Taz deficiency disrupts mitochondrial membrane composition and supercomplex integrity but not cardiac function. A, graphical summary (n = 6/group) and representative immunoblot of 30-kDa tafazzin protein detected in cardiac mitochondria from WT mice, but not Taz shRNA (TazKD) mice. B and C, fatty acid composition of cardiolipin (B) and total phospholipids (PL, C) extracted from SS and IF cardiac mitochondria (n = 4–6/group). D, representative images of high-molecular-mass protein complexes separated from WT and TazKD cardiac mitochondria by blue native (BN)–PAGE, subsequently probed for respiratory chain complexes I–V by SDS-PAGE (full analysis in Fig. S2). E, echocardiographic measures of left ventricular end-diastolic dimensions (EDD) and endsystolic dimensions (ESD) and corresponding fractional shortening (FS; (EDD − ESD)/EDD × 100) for 4–6-month-old WT and TazKD mice (n = 12/group). *, P < 0.01 for WT versus TazKD.

Figure 2. Substrate-specific impairment of OXPHOS capacity in Taz-deficient cardiac mitochondria. A, protein yield of SS and IF mitochondria isolated from WT and TazKD mouse hearts. B, spectrophotometric assessment of cytochrome c oxidase enzymatic activity from SS and IF mitochondria. C, state III respiration of isolated SS and IF cardiac mitochondria stimulated by 250 μM ADP, supported by the indicated substrates. D, membrane potential (∆Ψm) of isolated SS and IF mitochondria energized with succinate + rotenone determined by safranin O fluorescence quenching. E, maximal rates of LEAK (L, no ADP) and OXPHOS-linked respiration (P, 4 mM ADP-stimulated) in permeabilized cardiac muscle fibers supported by the indicated substrates. F, high-resolution respirometry substrate titration protocol generating multiple respiratory states in permeabilized cardiac fibers (see text for details). G, ADP respiratory control factors ([P − L]/L) for pyruvate + malate (PM), palmitoylcarnitine + malate (PalM), and glutamate + malate (GM) in WT and TazKD cardiac muscle fibers. The data are means ± S.E. (n = 6–8/group). *, P < 0.05 TazKD versus WT. Carn, carnitine; Glut, glutamate; Palm, palmitoylcarnitine; Pyr, pyruvate; Succ, succinate.
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complex I (NADH dehydrogenase; CI) and complex IV (cytochrome c oxidase; CIV) were 16 and 26% lower in Taz\textsuperscript{KD} versus WT mitochondria, respectively (Fig. 2B), whereas state III (ADP-stimulated) respiration rates supported by CI-dependent pyruvate and palmitoylcarnitine (+ malate) were 41–66% lower in Taz\textsuperscript{KD} versus WT (Fig. 2C). However, state III respiration supported by glutamate + malate, which is also CI-dependent, was 45–68% higher in Taz\textsuperscript{KD} compared with WT mitochondria, reaching rates similar to those achieved with pyruvate and palmitoylcarnitine in WT. State III respiration with succinate + rotenone (supplying electrons through succinate dehydrogenase; CII) far exceeded rates with other substrates and was slightly lower in Taz\textsuperscript{KD} versus WT mitochondria, whereas mitochondrial membrane potential was identical between cohorts (Fig. 2D).

To confirm that the results obtained from mitochondria were not artifacts of the isolation procedure, high-resolution respirometry experiments were performed on permeabilized cardiac fibers with intact mitochondrial matrices. Indeed, similar substrate-specific defects in maximal (5 mM) ADP-stimulated respiration rates with pyruvate and palmitoylcarnitine (+ malate) were 41–66% lower in Taz\textsuperscript{KD} versus WT (Fig. 2G). Consistent with these findings, high-molecular-mass (>90 kDa) 4-hydroxynonenal-protein adducts and protein carbonyls were greater in Taz\textsuperscript{KD} versus WT mitochondria isolated from freshly harvested hearts (Fig. 3, C and D), indicating chronic elevation of mitochondrial oxidative stress in vivo. The absence of evidence for oxidative damage in lower-molecular-mass mitochondrial proteins might indicate their tendency to destabilize or be degraded by Lon protease as discussed below.

Tafazzin deficiency disrupts cardiac mitochondrial CoA metabolism

Pyruvate and palmitoylcarnitine both require CoA to support OXPHOS in mitochondria, whereas glutamate + malate can be oxidized to generate NADH by CoA-independent reactions via the malate–aspartate shuttle (31). Therefore, we hypothesized that tafazzin deficiency may alter mitochondrial CoA metabolism or availability. Indeed, we found that levels of free CoA in cardiac mitochondria (Fig. 4A) and tissue (Fig. 4B) were 40–50% lower in Taz\textsuperscript{KD} than WT. Similarly, tissue levels of pantothenic acid, the essential B-vitamin precursor of CoA, were also 40% lower in Taz\textsuperscript{KD} compared with WT hearts (Fig. 4C). Total acyl-CoA content (pmol/mg protein) assessed by LC–MS was also lower in snap-frozen Taz\textsuperscript{KD} hearts (Fig. 4D), which mainly reflected lower levels of long-chain acyl-CoAs (C13–20) and acetyl-CoA (Fig. 4E). Relative to total tissue acyl-CoA content, the proportions of short- and medium-chain acyl-CoAs were greater in Taz\textsuperscript{KD} versus WT, whereas long-chain species tended to decline Fig. 4F. However, a 93% higher relative proportion of 3-hydroxy-16:0-CoA was seen in Taz\textsuperscript{KD} versus WT (Fig. 4G), suggesting a site-specific defect in long-chain fatty acid oxidation. A full listing of individual acyl-CoAs detected by LC–MS in Taz\textsuperscript{KD} and WT hearts is presented in Table S1.

Mitochondrial proteomics reveals distinct pattern of metabolic remodeling in tafazzin deficiency

To explore the basis for the observed effects of tafazzin deficiency on mitochondrial metabolism, we performed targeted LC–MS/MS proteomic profiling of WT and Taz\textsuperscript{KD} mitochondria isolated from freshly harvested hearts. A total of 158 mitochondrial proteins met strict criteria for confident identification against the mouse Uniprot protein database (FDR, 0.1%), 103 of which were sorted based on functional annotations linking them to respiratory complexes or major energy metabolism pathways (Table S2). The majority of these are presented in Fig. 5A for interpretive analysis. Comparison of combined spectral counts from subunits representing individual respiratory chain complexes revealed a significant (22%) loss of total CI protein in Taz\textsuperscript{KD} versus WT per mg of total protein (p < 0.05), but no significant differences in complexes II, III, and IV or ATP synthase (Fig. 5B). Among the 33 CI subunits detected, those localized to the proximal membrane domain or “heel” of the complex were collectively lower by ~20–40% in Taz\textsuperscript{KD} versus WT mitochondria (Fig. 5C). Subunits in the distal membrane domain and peripheral arm were relatively unaffected, with the exception of NDUF12 (B17.2) and NDUF9 (39 kDa), which link catalytic subunits in the arm to core subunits in the membrane domain (32, 33). This selective loss of CI subunits...
observed accumulation of 3-hydroxy-16:0-CoA in greater abundance.

The count of the pyruvate dehydrogenase complex proteins was lower in TazKD and the dienoyl-CoA reductase was not affected by tafazzin deficiency, reflecting a specific impairment of the inner membrane long-chain fatty acid metabolism (Fig. 5).

TFP-β catalyzes the CoA-thiolysis of long-chain keto acyl-CoAs generated by the α-subunit, representing the final reaction of the inner membrane long-chain fatty acid β-oxidation cycle (37). Interestingly, TFP-α also possesses monolyso-cardiolipin acyltransferase activity (38), which was 3.5-fold higher in TazKD versus WT (Fig. 3), and subsequent degradation by Lon protease (34), which might result in part from oxidation of CI proteins by mtROS (Fig. 5).

Several enzymes involved in fatty acid oxidation were differentially affected by tafazzin deficiency, reflecting a specific reorganization of acyl-CoA metabolism (Fig. 5E). In particular, TazKD mitochondria exhibited >50% lower levels of long-chain acyl-CoA synthetase and moderately (11%) lower levels of very-long-chain acyl-CoA dehydrogenase (p ≤ 0.05), consistent with impaired oxidation of long-chain fatty acids. Medium-chain acyl-CoA dehydrogenase (MCAD) and the dienoyl-CoA reductase were also 25–30% lower in TazKD, consistent with an accumulation of short- and medium-chain acyl-CoAs and specifically 10:1-CoA (Table S1). However, acyl-CoA thioesterases 2 and 13, both selective for long-chain acyl-CoAs (35, 36), were significantly (90%) higher in TazKD mitochondria (Fig. 5F), along with 20% greater levels of trifunctional protein-β (TFP-β) (Fig. 5E). TFP-β catalyzes the CoA-thiolysis of long-chain keto acyl-CoAs generated by the α-subunit, representing the final reaction of the inner membrane long-chain fatty acid β-oxidation cycle (37). Interestingly, TFP-α also possesses monolyso-cardiolipin acyltransferase activity (38), and the observed accumulation of 3-hydroxy-16:0-CoA in TazKD hearts suggests a specific block in the α-subunit’s β-oxidation function (37).

Despite markedly lower pyruvate oxidation capacity of TazKD versus WT mitochondria (Fig. 2), the combined spectral count of the pyruvate dehydrogenase complex proteins was greater in TazKD, largely because of 43% higher levels of the E2 subunit responsible for acetyl-CoA formation (Fig. 5G). Tricarboxylic acid cycle enzymes were not uniformly affected by tafazzin deficiency but collectively tended to increase because of the 40% higher levels of malate dehydrogenase (MDH) (Fig. 5H). Notably, MDH is responsible for NADH production from glutamate oxidation in the malate–aspartate shuttle (MAS) (31, 39), which was also ~40% higher in TazKD compared with WT mitochondria (Fig. 2). To determine whether tafazzin deficiency increases cardiac availability of amino acids as anaplerotic substrates, we performed targeted GC/MS metabolite analysis of snap-frozen cardiac samples from TazKD and WT mice. Indeed, all amino acids were higher in TazKD except for proline (lower) and glutamate (unchanged) (Fig. 5I), consistent with a greater utilization of glutamate to maintain anaplerotic flux in the face of impaired pyruvate and fatty acid oxidation. Taken together, these analyses corroborate our functional evidence for a selective defect in CoA metabolism and a rearrangement of substrate oxidation pathways favoring glutamate utilization in tafazzin-deficient cardiac mitochondria.

Exogenous CoA supplementation partially rescues fatty acid and pyruvate oxidation in TazKD mitochondria

To further investigate how tafazzin deficiency impairs fatty acid oxidation capacity, we evaluated OXPHOS rates of TazKD and WT mitochondria by substrates entering the pathway at specific sites (Fig. 6A). Palmitoylcarnitine bypasses carnitine palmitoyltransferase 1 (CPT1) for β-oxidation initially by the long-chain-specific system on the inner mitochondrial membrane (very-long-chain acyl-CoA dehydrogenase and TFP), followed by the medium- and short-chain acyl-CoA dehydrogenases in the canonical matrix β-oxidation cycle (Fig. 6B).
In *Taz^KD* mitochondria. There were no effects of CoA enrichment on WT mitochondrial function, suggesting that physiological CoA levels are sufficient to support maximal OXPHOS. Collectively, these studies implicate mitochondrial CoA availability as a primary limitation of maximal carbohydrate and fatty acid oxidation capacity in tafazzin-deficient cardiac mitochondria.

**Discussion**

This study sought to elucidate how impaired cardiolipin remodeling impacts cardiac mitochondrial function and identify potentially compensatory adjustments to tafazzin deficiency that manifest prior to development of cardiac dysfunction. The *Taz* shRNA mouse proved ideal for this purpose, exhibiting persistent depletion of L^4^CL and CI–CIII–CIV mitochondrial supercomplexes characteristic of BTHS (14, 41) and other cardiac pathologies (42, 43) but preserved cardiac function well into adulthood. The results confirmed moderate impairment of CI and CIV enzymatic capacities in *Taz^KD* mitochondria that parallel findings from BTHS patient–derived cell lines and are consistent with a loss of CI subunits (Fig. 5) and previous links between L^4^CL content and CIV activity (44, 45). However, isolated respiratory enzyme capacities far exceed the activity required to support maximal OXPHOS rates, such that moderate reductions might not impair intact mitochondrial energetics (46). Indeed, although maximal CI-dependent OXPHOS supported by pyruvate or palmitoylcarnitine (+ malate) was lower in *Taz^KD* CI-dependent glutamate + malate OXPHOS approximated rates obtained in WT with other CI substrates, implicating defects upstream of the respiratory chain. Similarly, succinate-supported OXPHOS was slightly lower in *Taz^KD*, consistent with a CII defect in this model (21), but multisubstrate CI+II-linked OXPHOS in permeabilized heart fibers was not significantly impaired. Therefore, although tafazzin deficiency clearly impairs respiratory enzyme capacity, these defects are evidently insufficient to limit integrated OXPHOS-linked respiratory capacity in intact mitochondria. These findings are consistent with normal basal rates of mitochondrial respiration, ATP synthesis, and tricarboxylic acid cycle flux recently reported from BTHS patient–derived cell lines (30) and suggest that mechanisms other than overt bioenergetic failure contribute to the pathogenesis of BTHS.

The CI-dependent assembly of respiratory supercomplexes has been postulated to improve electron channeling between individual complexes, such that disruption might impair OXPHOS efficiency or increase mtROS formation (47). We found no difference in the capacity of *Taz^KD* versus WT mitochondria to maintain high membrane potential in the absence of ADP (Fig. 2D), nor the degree of OXPHOS coupling control by ADP with any substrates (Fig. 2G), suggesting that tafazzin/cardiolipin deficiency and CI/III/IV supercomplex instability do not impair the efficiency of OXPHOS or proton pumping. However, mtROS release during OXPHOS was significantly greater from *Taz^KD* versus WT mitochondria and paralleled greater accumulation of mitochondrial protein carbonyls and 4-hydroxynonenal–protein adducts. Neither the addition of fatty acid or inhibition of CI by rotenone altered the magnitude

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**Figure 4. Taz deficiency disrupts cardiac acyl-CoA metabolism.** A and B, free CoA content of isolated cardiac mitochondria (MITO, A) and snap-frozen cardiac tissue (B) from WT and *Taz^KD* mice. C and D, panthothenic acid levels (C) and total acyl-CoAs (D) in cardiac tissue. E and F, acyl-CoAs grouped by number of carbons ranging from acetyl-CoA (C2) to 18–20 carbon acyl-CoAs (C18–20) expressed per mg tissue (E) and as a percentage of total acyl-CoAs (F). G, 3-OH-palmitoyl-CoA accumulates relative to total acyl-CoA content in *Taz^KD* versus WT. The data are means ± S.E. *p < 0.05 Taz^KD* versus WT.

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5A). Similar to results from SS and IF mitochondria and permeabilized fibers (Fig. 2), maximal palmitoylcarnitine-supported OXPHOS in a combined (SS + IF) mitochondrial fraction from *Taz^KD* hearts was ~58% of WT (p < 0.01; Fig. 6, A and B). Oxidation of C8 octanoylcarnitine, which bypasses CPT1 and the long-chain β-oxidation system for oxidation by MCAD, was similarly lower in *Taz^KD*, arguing against an isolated rate-limiting defect in long-chain fatty acid oxidation. Surprisingly, oxidation of palmitoyl-CoA + carnitine, which requires CPT1 activity, was not significantly lower in *Taz^KD* than WT, arguing against a rate-limiting defect in mitochondrial fatty acid transfer.

Given evidence for lower CoA levels in *Taz^KD* mitochondria, we hypothesized that exogenous supply of CoA might have contributed to the higher relative (*Taz^KD*/WT) oxidation rates with palmitoyl-CoA compared with acylcarnitines (Fig. 6B), because free CoA is readily transported into isolated cardiac mitochondria (40). Indeed, preincubation of mitochondria with CoA using an established protocol (40) increased levels ~2-fold in *Taz^KD* and WT mitochondria, restoring CoA in *Taz^KD* to untreated WT levels (Fig. 6C). This CoA enrichment increased OXPHOS-linked respiratory capacity supported by pyruvate + malate (ADP in Fig. 6D), palmitoylcarnitine + malate (ADP in Fig. 6E), and their combination (Pyr in Fig. 6E)
of this effect, suggesting that site(s) downstream of CI in the respiratory chain are the primary source of greater mtROS release from \( \text{Taz}^{KD} \) mitochondria. We postulate that impaired linkage of CI to CIII–CIV supercomplexes drives greater mtROS generation and an accumulation of oxidative damage that ultimately impairs cardiac function in \( \text{Taz}^{KD} \) mice by 8–10 months of age (24, 25), consistent with the mechanistic role of excess ROS in cardiomyocyte dysfunction demonstrated by in vitro models of tafazzin deficiency (18, 48).

The healthy mammalian heart relies almost exclusively on the oxidation of long-chain fatty acids and pyruvate to meet its energy demands (49). However, cardiomyocytes also readily oxidize glutamate via the MAS, which plays a key role in coordinating cytosolic and mitochondrial redox balance and energy homeostasis (50), particularly under states of metabolic stress (e.g. ischemia reperfusion) (31). MAS activity is governed largely by the availability of glutamate and \( \alpha \)-ketoglutarate dehydrogenase (OGDH) activity, whereby reduced flux through OGDH directs \( \alpha \)-ketoglutarate to the MAS, facilitating uptake and oxidation of malate by MDH (50). The up-regulation of glutamate oxidation capacity in \( \text{Taz}^{KD} \) mitochondria herein was associated with lower expression of the Dlst (CoA-binding) subunit of the OGDH complex and higher MDH expression, consistent with a compensatory shift in tricarboxylic acid flux to favor higher MAS activity. This finding is corroborated by a specific destabilization of the OGDH complex in human BTHS cells, without impairment of cellular respiration or ATP production (30). We postulate that these changes reflect an adaptive reorganization of oxidative pathways to compensate for deficient pyruvate and fatty acid oxidation in tafazzin-deficient mitochondria, perhaps partially offsetting the bioenergetic stress of suboptimal cardiac fuel utilization. Indeed, greater capacity for systemic proteolysis and amino acid oxidation is associated with reduced cardiac systolic strain in BTHS patients (51). It is also noteworthy that the brain exhibits constitutively high MAS activity and glutamate oxidation capacity (52) and is relatively unaffected in BTHS despite being a primary locus of pathology in other mitochondrial disorders (53).

Elucidating how tafazzin deficiency impairs oxidation of fatty acids and pyruvate, but not glutamate, is of considerable
scientific and clinical interest, because this remains a severe li-
mitation to optimal cardiac bioenergetics in BTHS (54). Results
from the present study suggest a potential block in medium-
chain acyl-CoA oxidation in \textit{TazKD} mitochondria, given evi-
dence for similar impairment of both long- and medium-chain
acylcarnitine oxidation, lower MCAD expression, and a relative
accumulation of C3-C12 acyl-CoAs. Protein levels of the pyru-
vate dehydrogenase complex subunits were not lower in
\textit{TazKD}, but this enzyme complex has been previously shown to be
inhibited by post-translational modifications in this and other
\textit{Taz}-deficient models (55). However, partial rescue of both pal-
mitoylcarnitine- and pyruvate-supported OXPHOS flux with
exogenous CoA herein suggests additional limitations imposed
by free CoA availability or impaired CoA-dependent reactions.
Up-regulation of long-chain–specific ACOT 2 and 13 could
reflect mitochondrial CoA “trapping” by incompletely oxidized
acyl-CoAs, perhaps resulting from a block in long-chain fatty
acid oxidation by TFP indicated by accumulation of 3OH-16:0-
CoA and up-regulation of its \(\beta\)-subunit (37). TFP requires free
CoA for activity and also catalyzes transfer of acyl-CoA to
monolysocardiolipin (38, 56), which accumulates in \textit{TazKD} mit-
tochondria, potentially interfering with its \(\beta\)-oxidation func-
tion. Whether the global decrease in mitochondrial CoA-esters
reflects an impairment or compensatory down-regulation of
CoA metabolism is unclear, but several aspects of the BTHS
phenotype align with systemic CoA/pantothenate deficiency
(57). Within this context, the therapeutic value of pantothenate
supplementation has been explored in BTHS patients with
mixed success, perhaps reflecting defects in the uptake or utili-
zation of B vitamins (57, 58). Our CoA rescue experiments
demonstrate \textit{Taz}-deficient mitochondria retain the ability to
import CoA, at least \textit{in vitro}, further implicating an upstream
decrease in CoA biosynthesis and/or pantothenate availability
that merits future investigation. Although the mechanism of
CoA deficiency remains unclear, we postulate that defects in
mitochondrial handling of acyl-CoAs play a central role in the
metabolic defects induced by tafazzin deficiency. The success-
ful up-regulation of CoA-independent glutamate oxidation in
\textit{TazKD} mice strongly supports this hypothesis, despite evidently
being insufficient to meet bioenergetic demands in BTHS
patients (54).

In summary, our studies reveal that defective cardiolipin me-
tabolism resulting from tafazzin deficiency selectively impairs
pyruvate and fatty acid oxidation but elicits compensatory up-
regulation of glutamate oxidation by the MAS that is capable of
supporting normal rates of OXPHOS-linked respiration in car-
diac mitochondria. This occurs despite expected reductions in
respiratory enzyme capacities and supercomplex instability,
implicating upstream defects in specific substrate oxidation
pathways as the primary limitation of normal bioenergetics in

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**Figure 6. Exogenous CoA partially rescues respiratory function of tafazzin-deficient cardiac mitochondria.** A and B, OXPHOS-linked respiratory capacity of cardiac mitochondria supported by CPT1-independent long-chain (palmitoyl; Palm) or medium-chain (octanoyl; Oct) acylcarnitines, or CPT1-dependent palmitoyl-CoA + carnitine expressed as O2 flux/mg protein (A), and relative activity between genotypes (\textit{TazKD}/\textit{WT}; B) \((n = 4\text{-}5\text{/group})\). C–E, exogenous CoA incu-
bation (+ CoA) increases mitochondrial CoA content in \textit{TazKD} mitochondria (C) and partially restores OXPHOS capacity of \textit{TazKD} mitochondria fueled by palmitoylcarnitine + malate (ADP in D) or pyruvate + malate (ADP in E) in cumulative substrate titration respirometry protocols. The data are means ± S.E. \((n = 4\text{-}6\text{/group})\). *, \(P < 0.05\) \textit{TazKD} versus all groups. #, \(P < 0.05\) \textit{TazKD} versus \textit{TazKD} + CoA. Carn, carnitine; Glut, glutamate; Pyr, pyruvate; Succ, succinate.
tafazzin-deficient hearts. Depletion or “trapping” of mitochondrial CoA secondary to disruptions of acyl-CoA metabolism likely contribute to this phenotype, with potential implications for the future investigation and treatment of BTHS. However, early and persistent elevations in mtROS release and oxidative stress may ultimately supersede bioenergetic defects in the pathogenesis of cardiomyopathy, consistent with previous reports from other BTHS models. Taken together, these studies provide new insights and perspective on how tafazzin and cardiolipin metabolism influence mitochondrial function in the mammalian heart, which should be considered along with advances contributed by lower-order and in vitro model systems.

Experimental procedures

Animal model

Doxycycline-inducible Taz shRNA (TazKD) mice, originally generated at TaconicArtemis, GmbH (Köln, Germany) under contract from the Barth Syndrome Foundation, were obtained from Dr. Zaza Khuchua at the University of Cincinnati (24) and used to establish a colony of TazKD and WT mice at Colorado State University for these studies. Both TazKD and WT mice were maintained on RMH1500 chow (Purina, Gray Summit, MO) supplemented with 625 mg/kg doxycycline to induce Taz shRNA expression and control for any unintended effects of doxycycline in WT mice. Transgenic males were mated with WT females, and the offspring were genotyped by PCR analysis previously described (60). Permeabilized fiber bundles were isolated from left ventricular sections and permeabilized with saponin as described (59). Mitochondria were freshly isolated from ~75–90 mg of heart tissue by differential centrifugation methods previously described (59). Mitochondrial preparations

Mitochondria were freshly isolated from ~75–90 mg of heart tissue by differential centrifugation methods previously described (59). For some experiments, SS and IF mitochondria were isolated using trypsin to dissociate IF mitochondria from myofibrillar proteins (59). To evaluate the respiratory function of intact cardiac mitochondrial matrices in situ, structurally sound fiber bundles (1.0–1.2 mg) were dissected from 10–15 mg left ventricular sections and permeabilized with saponin as previously described (60). Mitochondrial phospholipid analyses

The fatty acid composition of total mitochondrial phospholipids, cardiolipin, phosphatidylcholine, and phosphatidylethanolamine was performed in lipids extracted from SS and IF mitochondrial fractions as previously described (59). Briefly, phospholipid classes were separated by normal phase liquid chromatography using a hexane:isopropanol:potassium acetate mobile phase gradient optimized for separation of phosphatidylethanolamine, phosphatidylcholine, and cardiolipin by UV detection (206 nm). Fractions were collected based on elution time of known standards, evaporated under a nitrogen stream, and resuspended in hexane, followed by the addition of 14% BF3-methanol and heating at 100 °C for 30 min to obtain methyl esters for gas chromatographic analysis of fatty acid composition. Cardiolipin molecular species were analyzed by electrospray ionization MS as previously described in detail (61).

Mitochondrial supercomplexes

High-molecular-mass mitochondrial enzyme complexes were separated in their native state by blue native–PAGE using 4–15% polyacrylamide gradient Tris–glycine gels (Bio-Rad, Criterion TGX) for 1 h at 70 volts with blue cathode buffer to image and quantitate density of supercomplex bands. High-molecular-mass bands (>500 kDa) were excised, treated with Laemmli buffer and 2-mercaptoethanol, and loaded on to 4–20% Tris–HCl gels for separation of individual respiratory chain complex subunits by SDS-PAGE. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes and blotted for OXPHOS complex levels using an antibody mixture determined by BCA assay. Echocardiography

Transthoracic echocardiography was performed under light (1.5%) isoflurane anesthesia using a 15-MHz linear array transducer connected to a Philips HD11 ultrasound. Short-axis 2D images of left ventricular end-diastolic and end-systolic areas were obtained for assessment of left ventricular chamber morphology and fractional shortening ((end-diastolic area − end-systolic area)/end-diastolic area) × 100). The data were averaged from four consecutive high-resolution cycles by the same technician.

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Mitochondrial respirometry

State 3 (ADP-dependent) respiration corresponding to OXPHOS was assessed in freshly isolated mitochondria (75 μg of protein) at 37 °C in response to 50 μM ADP at an initial
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氧浓度为150 μM的O2k高分辨率呼吸仪（Oroboros Instruments, Innsbruck, Austria）。多个实验分别在MiR06缓冲液(60)中进行，使用 saturating concentrations of the following substrate combinations: pyruvate (5 mM) + malate (1 mM), palmitoylcarnitine (0.04 mM) + malate (1 mM), succinate (20 mM) + rotenone (100 μM), or malate (1 mM) + glutamate (10 mM). Oxygen flux was monitored in real time by resolving changes in the negative time derivative of the chamber oxygen concentration signal following standardized instrumental and chemical background calibrations using DataLab software (Oroboros Instruments). Respirometry studies in permeabilized fibers were conducted in a hyperoxygenated environment (275–400 μM) to avoid limitations in oxygen diffusion (62, 63) under the identical buffer and substrate conditions described above. For fibers and some mitochondrial studies, substrates were added in series as indicated under “Results,” and 5 mM ADP was used to obtain maximal OXPHOS capacities under cumulative substrate conditions.

Mitochondrial enzyme activities and membrane potential

Enzymatic activities of respiratory complexes I and IV were determined in frozen-thawed mitochondrial isolates by monitoring the oxidation of NADH or cytochrome c, respectively, by previously described spectrophotometric methods (61) using a Spectramax M2e spectrophorometer (Molecular Devices) normalized to total protein by the BCA assay (Pierce). Relative enzymatic activities of Respiratory complexes I and IV were determined using saturating concentrations of the following substrate combinations: pyruvate (5 mM) + malate (1 mM), palmitoylcarnitine (0.04 mM) + malate (1 mM), succinate (20 mM) + rotenone (100 μM), or malate (1 mM) + glutamate (10 mM). Oxygen flux was monitored in real time by resolving changes in the negative time derivative of the chamber oxygen concentration signal following standardized instrumental and chemical background calibrations using DataLab software (Oroboros Instruments). Respirometry studies in permeabilized fibers were conducted in a hyperoxygenated environment (275–400 μM) to avoid limitations in oxygen diffusion (62, 63) under the identical buffer and substrate conditions described above. For fibers and some mitochondrial studies, substrates were added in series as indicated under “Results,” and 5 mM ADP was used to obtain maximal OXPHOS capacities under cumulative substrate conditions.

Mitochondrial enzyme activities and membrane potential

The net rate of mitochondrial H2O2 release was determined during respirometry experiments by monitoring the accumulation of chamber resorufin (excitation/emission, 525/575 nm), the stable fluorescent product of 1:1 oxidation of Amplex UltraRed (AmR, Molecular Probes; 5 μM) by H2O2 in the presence of horseradish peroxidase (1 units/ml) (64) in our Oroboros respirometer. Resorufin fluorescence was calibrated to chamber [H2O2] using freshly prepared H2O2 standards (0.1–1 μM) stabilized with 10 μM HCl as previously described (65) and corrected for chemical background reactions in MiR05 respiration buffer (MiR06 without catalase) in the presence of respiratory substrates: 1 mM malate, 5 mM pyruvate, 10 mM glutamate, and 20 mM succinate, with or without 2.5 mM ADP. All data were collected between 180 and 200 μM O2 to avoid confounding effects of different chamber O2 concentrations on mtROS production. To assess the extent of chronic oxidative stress to mitochondria, 30 μg of mitochondrial protein were immuno-blotted for 4-hydroxynonenal–protein adducts (Calbiochem; catalog no. 393207; 1:2000 dilution) and protein carboxyls (Oxyblot; Millipore Sigma S7150) quantified by densitometry (ImageJ, National Institutes of Health) following chemiluminescent imaging of bands using a UVP ChemiDoc imager (Upland, CA, USA).

Cardiac tissue and mitochondrial CoA content and supplementation studies

Mitochondrial CoA content was determined in isolated mitochondria using a commercially available colorimetric assay kit (BioVision; catalog no. K367-100) and normalized to mitochondrial protein by BCA assay. Acyl-CoAs were quantified in cardiac tissue homogenates by LC–MS using the method of Palladino et al. (66) on an API 4000 electrospray ionization mass spectrometer and an Acuity UPLC HILIC column (Waters, Milford, MA). To evaluate whether mitochondrial CoA content limited respiratory capacities of isolated mitochondria, respiratory studies were performed in mitochondrial suspensions preincubated with CoA as previously described to induce uptake and accumulation of CoA into the matrix of isolated rat cardiac mitochondria (40). Briefly, mitochondrial suspensions (200 μg of protein) freshly isolated from the same mouse heart were incubated in 200 μl of KME suspension buffer (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA, pH 7.4) containing 8 mM ATP with or without 100 μM CoA at 30°C for 20 min under gentle shaking. Following incubation, mitochondrial suspensions were cooled on ice and pelleted at 3000 × g at 4°C, resuspended in 200 μl of KME, and repelleted. The resulting mitochondrial pellets were washed and resuspended in 50 μl of KME, assayed for total protein and CoA content, and immediately used in respiration experiments as described above.

Mitochondrial proteomic profiling

Targeted LC–MS/MS analyses of mitochondrial proteins was performed in 30 μg of protein extracted from cardiac IF mitochondria isolated from TazKΔ mice and WT mice (n = 3/group). Trypsin-digested samples were subjected to offline UPLC-UV fractionation and concentration for LC–MS/MS analysis on a Thermo Scientific LTQ linear ion trap mass spectrometer. MS/MS spectra were searched against the mouse Uniprot protein database concatenated with reverse sequences for determination of the peptide FDR (118,690 sequence entries; 0.1%) using both the MASCOT database search engine (version 2.3) and Sorcerer™.SEQUEST® (67). A detailed description of the proteomic profiling and data analysis methods is provided in the supporting information.

Cardiac amino acid and pantothenate analysis

Six TazKΔ and WT mice were deeply anesthetized with sodium pentobarbital prior to rapid midline thoracotomy and freeze clamping of the beating heart within 10–15 s using a hemostat precooled in liquid nitrogen. The resulting ~30 mg frozen heart wafer was homogenized in 1 ml of methanol/water (70:30) and processed for GC/MS analysis using a Trace GC Ultra gas chromatograph coupled to a Thermo DSQ II mass spectrometer (Thermo Scientific) as described in detail in the supporting information. Molecular features were formed into peak groups using AMDIS software, and the spectra were screened in the National Institute for Technology Standards (www.nist.gov) and Golm (http://gmd.mpimp-golm.mpg.de/)
metabolite databases for identifications, validated comparing retention indices and mass spectra from heart extracts to databases of authentic standards (68, 69).

Statistical analyses

All data are presented as group means ± S.E. Group differences between TazKD and WT were examined by independent sample t tests with Bonferroni–Hochberg corrections for multiple comparisons (FDR, 0.05) for metabolite and proteomic where appropriate for pairwise comparisons. Analyses of variance were used to determine significant differences between greater than two groups, followed by Tukey HSD post hoc test where appropriate for pairwise comparisons.

Data availability

Raw MS data are available online at 10.5281/zenodo.3932695.

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Abbreviations—The abbreviations used are: BTHS, Barth syndrome; CL, cardioplin; L-CL, tetra-lysineooyl cardioplin; shRNA, short hairpin RNA; SS, subsarcolemmal; IF, intermyofibrillar; OXPHOS, oxidative phosphorylation; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; mtROS, mitochondrial reactive oxygen species; FDR, false discovery rate; MCAD, medium-chain acyl-CoA dehydrogenase; TPF, trifunctional protein; MDH, malate dehydrogenase; MAS, malate–aspartate shuttle; CPT, carnitine palmitoyltransferase; OGDH, α-ketoglutarate dehydrogenase.

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