Tafazzin knockdown causes hypertrophy of neonatal ventricular myocytes

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Tafazzin knockdown causes hypertrophy of neonatal ventricular myocytes. Am J Physiol Heart Circ Physiol 299: H210–H216, 2010. First published March 26, 2010; doi:10.1152/ajpheart.00098.2010.—Mutation of the mitochondrial protein tafazzin causes dilated cardiomyopathy in Barth syndrome. We employed an adenvirus as a vector to transfer tafazzin small hairpin RNA (shRNA) into neonatal ventricular myocytes (NVMs) to investigate the effects of tafazzin knockdown. The tafazzin shRNA adenosvirus consistently knocked down tafazzin mRNA and lowered cardiolipin while significantly decreasing the production of ATP by the mitochondria. The phosphorylation of AMP-activated protein kinase and mitochondrial density were both increased in tafazzin knockdown NVMs compared with scrambled shRNA controls. When we tested whether tafazzin knockdown causes hypertrophy in vitro, we found that the surface area of NVMs infected with tafazzin shRNA adenosvirus was significantly increased, as were the protein synthesis and expression of the hypertrophic marker gene, brain natriuretic peptide. Taken together, our data support the concept that a decreased tafazzin expression causes cardiomyocyte hypertrophy in vitro.

cardiolipin; adenosine 5′-triphosphate; dilated cardiomyopathy

TAFazzin is a mitochondrial protein encoded by the G4.5 gene in humans (7). It is a phospholipid transacylase (54), and its mutation results in decreased cardiolipin (18, 47–49, 55), a phospholipid predominantly present in the inner membrane of the mitochondria. Cardiolipin is initially synthesized as a precursor form, which is deacetylated by a phospholipase to generate monolysocardiolipin, and this lipid is finally reacylated by tafazzin to form the mature fully functional cardiolipin (17, 54). Cardiolipin is required for 1) optimal activity of respiratory chain complexes and ADP-ATP translocase (31), which are involved in ATP and reactive oxygen species production; 2) cytochrome c attachment within the mitochondrial intermembrane space (39), which is associated with mitochondria-initiated cell death; 3) maintaining inner membrane fluidity and osmotic stability (11), which are associated with the opening of mitochondrial permeability transition pores and mitochondria-gated cell death; and 4) mitochondrial protein import (30), which is associated with mitochondrial biogenesis.

Tafazzin mutation causes Barth syndrome, a rare X-linked genetic disorder characterized by dilated cardiomyopathy (DCM) with or without hypertrophy, skeletal myopathy, neutropenia, growth retardation, and 3-methylglutaconic aciduria (4, 5, 45). End-stage heart failure resulting from DCM is a major cause of death among these patients during infancy and early childhood.

DCM, the most common type of cardiomyopathy, is characterized by ventricular chamber enlargement and systolic dysfunction, commonly resulting in congestive heart failure (35). An eccentric cardiomyocyte hypertrophy (increased length-to-width ratio) and an energy depletion are characteristic of DCM (15, 20, 21, 36).

A deletion of tafazzin in yeast causes mitochondrial dysfunction, including changes in energy transformation and osmotic properties of the mitochondria (18, 34). A mutation of tafazzin in Drosophila causes a pathological dysfunction of mitochondria and motor weakness (53). Tafazzin knockdown in zebrafish led to bradycardia and retarded cardiac development (32). The decreased expression of tafazzin has been shown in spontaneously hypertensive failing hearts (42). However, we do not know how tafazzin knockdown affects mammalian cardiomyocytes. We here present evidence that tafazzin knockdown results in ATP deficiency and hypertrophy in cultured neonatal cardiomyocytes.

METHODS

All animal experiments were approved by the Henry Ford Health System Institutional Animal Care and Use Committee.

Supplies and chemicals. Phosphatase and proteinase inhibitor cocktail tablets (PhosSTOP and Complete Mini) were obtained from Roche Applied Science (Indianapolis, IN). A pSilencer adeno 1.0-CMV system was purchased from Ambion (Austin, TX). Primary antibodies against phospho-AMP-activated protein kinase (phospho-AMPK) (Thr172) and AMPK and an horseradish peroxidase-conjugated secondary antibody against rabbit IgG were obtained from Cell Signaling Technology (Boston, MA). β-Actin antibody and FITC-conjugated anti-goat IgG were purchased from Santa Cruz (Santa Cruz, CA). Coomassie gel stain assay and SuperSignal West Pico chemiluminescent substrate kits and Restore Plus Western blot stripping buffer were purchased from Thermo Scientific (Rockford, IL). MitoTracker Red, Taq DNA polymerase kits, DMEM medium and cell culture supplements, precast Tris-glycine polyacrylamide gels, and polyvinylidene fluoride membranes were obtained from Invitrogen (San Diego, CA). Laminin-coated coverslips were purchased from BD Sciences (San Jose, CA). ATP bioluminescent assay kit and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma (St. Louis, MO). [32P]phosphate and [3H]leucine were purchased from PerkinElmer (Waltham, MA); Whatman TLC plates (LKD silica gel, 150 Å) were from VWR (Batavia, IL); RNaseasy fibrous tissue mini kits, QIAamp DNA Micro Kit, random primers, and OmniScript reverse transcriptase were from Qiagen (Valencia, CA); and SYBR green dye was from SA Biosciences (Frederick, MD). Custom primers were designed by TIB MolBiol (Adelphia, NJ). The HEK293 cell line was obtained from ATCC (Manassas, VA), luciferase assay reagents were from Promega (Madison, WI), and other routine supplies and chemicals were from Fisher and Sigma.

Construction of tafazzin small hairpin RNA adenosvirus. The small hairpin sequences recognize tafazzin coding regions 5′-CACGT-GTGGAGATCGCGGA-3′ Taz1 and 5′-AGAGGAATCCAGCG-GCT-3′ Taz2 with restriction endonuclease XhoI and SpeI sites added at each end to facilitate subcloning. The small hairpin RNA (shRNA) oligonucleotides and scrambled sequence 5′-CACGTCAAGTGGACTTCT-3′ were synthesized by TIB MolBiol. Equal amounts of the upper and lower oligonucleotide were annealed by boiling and cooled to make the double-stranded shRNA. This was subcloned into a shuttle vector (pSilencer adeno 1.0-CMV), and the insertion was confirmed by sequencing. The virus DNA containing the insertion was packaged inside...
HEK293 cells. The adenovirus was expanded and the virus concentration (expressed as viral particles per milliliter) was estimated by spectrophotometry following Ambion’s protocol.

Neonatal cardiac myocyte culture. Neonatal ventricular myocytes (NVMs) were isolated from 1- to 2-day-old Sprague-Dawley rats from Charles River as described previously (24). They were plated on six-well (1 million cells per well) to improve the visualization of each individual cell. After we treated them with the adenovirus, they were immunostained with a β-actin antibody and visualized with an FITC-conjugated secondary antibody. The nuclei were counterstained by DAPI. Five images of each field were captured with fluorescence microscopy using DAPI and FITC filters and merged with SPOT software. The surface area (expressed as μm²) was measured with MicroSuite software.

[3H]leucine incorporation assay. The rate of protein synthesis by NVMs was estimated by [3H]leucine incorporation as described by Harding et al. (21). NVMs were plated on six-well plates at a density of one million cells per well. After a 40-h incubation in DMEM containing 10% FBS, the medium was changed to serum-, glucose-, and pyruvate-free DMEM containing 1 μCi [3H]leucine, and the adenovirus was added. After 48 h, the cells were harvested for trichloroacetic acid precipitates, which were counted for [3H] activity (counts/min of [3H]leucine incorporation) in a scintillation counter.

Mitochondrial staining. NVMs were transfected by electroporation using a cuvette with a 2-mm gap (BTX, San Diego, CA) in 0.4 ml PBS buffer containing 0.1% glucose and 1 μg –1818hBNPluciferase construct (33) was graciously provided by Dr. M. C. LaPointe (Henry Ford Hospital, Detroit, MI).

RESULTS

Construction of tafazzin shRNA adenovirus. To test the efficiency of our tafazzin shRNA adenovirus in knocking down tafazzin mRNA, we used doses ranging from 0.1 to 100 viral particles per myocyte (vp/cell) of two different constructs (Taz1 and Taz2). We found that 100 vp/cell gave the best results without affecting cell growth (Fig. 1A). Some NVMs were killed and the others displayed cell abnormal morphology when treated with 800 vp/cell shRNA adenovirus (data not shown). Whereas both Taz1 and Taz2 significantly knocked down tafazzin mRNA, Taz1 reduced the expression by 70% compared with only 28% for Taz2 (Fig. 1B). The internal control gene GAPDH was not affected by the shRNA adenovirus. Based on our findings, we used a dose of 100 vp/cell Taz1 shRNA adenovirus for all experiments.

Tafazzin knockdown decreases cardiolipin. Tafazzin mutation decreases cardiolipin content (18, 43, 48). We then tested whether tafazzin knockdown in NVMs affects cardiolipin content and found that tafazzin knockdown significantly decreased cardiolipin in NVMs (Fig. 2) compared with scrambled con-
control, as determined by metabolic labeling and thin-layer chromatography analysis. In contrast, other phospholipids were not affected by tafazzin knockdown. Thus tafazzin knockdown in NVMs mimics cardiolipin deficiency in Barth syndrome.

Tafazzin knockdown decreases intracellular ATP. The primary function of the mitochondria is the production of ATP, which is critically important for maintaining the pumping function of the heart. ATP depletion has been associated with cardiomyopathy and heart failure (2, 3, 8, 36). Barth syndrome, which is characterized by DCM, causes metabolic decomposition and energy deficiency (12, 34, 56). We infected NVMs with the tafazzin shRNA adenovirus for 48 h in a medium containing glutamine as the sole energy molecule and found that intracellular ATP was significantly reduced compared with cells infected with a scrambled shRNA adenovirus (Fig. 3). This ATP must have come from the mitochondria, because glutamine must be metabolized by oxidative phosphorylation within the mitochondria to generate ATP (38); thus tafazzin knockdown decreases mitochondrial ATP production.

Tafazzin knockdown activates AMPK and increases mitochondrial density. AMPK has been postulated as the “fuel gauge” of the cell and is regulated by intracellular ATP (19). It is activated by a decrease in the ATP-to-AMP ratio. One of the long-term responses to AMPK activation is an enhanced mitochondrial biogenesis (29). We investigated the effects of tafazzin on AMPK activation and mitochondrial biogenesis and found that tafazzin knockdown dramatically increased AMPK phosphorylation (Fig. 4A) compared with a scrambled virus control. As visualized by MitoTracker red (Fig. 4, B and C), the mitochondrial density (red stains) around the nucleus, which was stained in blue by DAPI, was significantly in-

Fig. 1. Tafazzin mRNA is knocked down by the tafazzin small hairpin (shRNA) adenovirus. A: representative images obtained by semiquantitative RT-PCR. Tafazzin mRNA was knocked down by the tafazzin shRNA adenovirus in a dose-dependent manner. TAZ, amplified tafazzin gene (278 bp); GAPDH, 302 bp. Lanes 1, 4, 7, and 10 represent neonatal ventricular myocytes (NVMs) treated with the scrambled virus; lanes 2, 5, 8, and 11 represent NVMs treated with the Taz1 shRNA adenovirus; and lanes 3, 6, 9, and 12 represent NVMs treated with the Taz2 shRNA adenovirus. MM, molecular marker; vp, viral particles. B: quantification of A. Tafazzin mRNA was expressed as a percentage of cells infected with a scrambled shRNA adenovirus (SCR). Data represent means ± SE from 14 independent experiments. #P < 0.01 vs. SCR; *P < 0.05 vs. SCR.

Fig. 2. Tafazzin shRNA adenovirus decreases cardiolipin in NVMs. A: TLC image representing 3 separate experiments. NVMs were infected with the shRNA adenovirus and metabolically labeled with [32P]phosphate. Phospholipids were isolated and analyzed on TLC plates as described in METHODS. Taz, tafazzin shRNA; CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine. B: quantification of cardiolipin from A. *P < 0.05 vs. SCR; n = 3 separate experiments.

Fig. 3. Tafazzin knockdown decreases intracellular ATP. NVMs were treated, harvested, and assayed for ATP as described in METHODS. ATP content was expressed as relative light units per microgram protein. Data represent means ± SE from 4 separate experiments. *P < 0.05 vs. SCR.
creased. The concentration of mitochondrial DNA is proportional to the number of mitochondria per cell (37). An enhanced mitochondrial biogenesis was further confirmed by the increased ND1 gene content as assayed by real-time PCR (Fig. 4D). These data suggest that decreased mitochondrial ATP production activates AMPK, which in turn enhances mitochondrial biogenesis.

**Tafazzin knockdown causes cardiac myocyte hypertrophy.**

We next studied the effect of tafazzin knockdown on NVM hypertrophy. We found that tafazzin knockdown significantly increased NVM size compared with the SCR control (Fig. 5, A and B). Protein synthesis as represented by [3H]leucine incorporation was also significantly increased (Fig. 5C).

We also investigated whether tafazzin knockdown induces the hypertrophic marker gene, BNP expression. As shown in Fig. 6, A and B, tafazzin knockdown significantly increased the expression of BNP as determined by semiquantitative RT-PCR. The activation of BNP gene transcription was assayed by measuring a hBNP promoter coupled to a luciferase reporter gene. Tafazzin knockdown significantly increased BNP promoter activity compared with the scrambled virus control (Fig. 6C). Thus tafazzin knockdown induced hypertrophy in NVMs.

**DISCUSSION**

Ablation or decreased expression of tafazzin has been shown to reduce cardiolipin in several types of cells and model systems (18, 43, 44, 47, 48, 53). Our current finding that tafazzin knockdown decreases cardiolipin in NVMs is consistent with previous cell studies. Cardiolipin deficiency due to tafazzin knockdown resulted in an impaired mitochondrial function (seen as reduced ATP production) as observed in yeast (34), as well as the energy depletion seen in patients with Barth syndrome.

The depletion of ATP in cardiac myocytes has several detrimental effects, including contractile dysfunction, hypertrophy, and cell death. Acutely, ATP depletion switches on the ATP-producing catabolic pathways such as fatty acid oxidation and glycolysis and switches off the ATP-consuming anabolic pathways such as lipogenesis and glycogenogenesis (41). One long-term response to ATP depletion is enhanced mitochondrial biogenesis, which is mediated by the activation of the “fuel gauge” AMPK (19). Jager et al. (29) reported that AMPK directly phosphorylates and activates PGC-1α, the key regulator of mitochondrial biogenesis. During strength and endurance training, more ATP is consumed, and this coincides with increased mitochondrial volume in the muscle (40). Our data showing that tafazzin knockdown increased mitochondrial density in NVMs support the concept that ATP depletion enhances mitochondrial biogenesis. This is also consistent with the increased number of mitochondria found in the lymphocytes of patients with Barth syndrome (55).

Our data show that tafazzin knockdown causes NVM hypertrophy as indicated by increased cell surface area, protein synthesis, and expression of the hypertrophic marker gene BNP. Several factors may be involved in the cardiomyocyte hypertrophy induced by tafazzin knockdown. First, increased...
mitochondrial density contributes to increased NVM volume because cardiac myocytes have a very high mitochondrial density (16), occupying about 40% of the cytoplasmic volume in mice (13). Previous studies also showed that mitochondrial volume and density increase in proportion to increased cell volume (26, 28, 46). Second, the depletion of ATP may directly cause cardiomyocyte hypertrophy, since ATP deficiency reduces contractile efficiency and this leads to compensatory hypertrophy. Finally, as we observed in yeast (9), impaired mitochondrial function due to a deletion of tafazzin causes increased oxidative stress that contributes to cardiomyocyte hypertrophy (1, 6, 9, 10, 27, 52). Without conducting pressure loading, we could not differentiate between eccentric (increased length-to-width ratio) and concentric hypertrophy (increased cross-sectional area); however, eccentric cardiomyocyte hypertrophy is characteristic of DCM based on pre-
vious studies (14, 15, 20). The fact that tafazzin knockout induces hypertrophy of NVMs is relevant in vivo because cardiac myocytes occupy 75% of the myocardial structural space even though they constitute only one third of the cell population (50). Undoubtedly, cardiomyocyte hypertrophy is a major factor in cardiomyopathy.

In summary, our data indicate that tafazzin knockout causes hypertrophy in NVMs. Decreased tafazzin expression lowers cardiolipin in NVMs, and this in turn blunts ATP production by the mitochondria. The decrease in ATP activates AMPK and enhances mitochondrial biogenesis, which contributes to the increased volume of NVMs. We believe our findings establish a link between tafazzin mutation and cardiomyocyte hypertrophy.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are disclosed by the author.

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