Expression of human monolysocardiolipin acyltransferase-1 improves mitochondrial function in Barth syndrome lymphoblasts

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The mitochondrial polyglycerophospholipid cardiolipin (CL) is remodeled to obtain specific fatty acyl chains. This is predominantly accomplished by the transacylase enzyme tafazzin (TAZ). Barth syndrome (BTHS) patients with TAZ gene mutations exhibit impaired TAZ activity and loss in mitochondrial respiratory function. Previous studies identified monolysocardiolipin acyltransferase-1 (MLCL AT-1) as a mitochondrial enzyme capable of remodeling CL with fatty acid. In this study, we analyzed what relationship, if any, exists between TAZ and MLCL AT-1 with regard to CL remodeling and whether transfection of BTHS lymphoblasts with MLCL AT-1 expression construct improves mitochondrial respiratory function. In healthy lymphoblasts, reduction in TAZ expression through RNAi transfection resulted in a compensatory increase in MLCL AT-1 mRNA, protein, and enzyme activity, but CL mass was unaltered. In contrast, BTHS lymphoblasts exhibited decreased TAZ gene and protein expression but in addition decreased MLCL AT-1 expression and CL mass. Transfection of BTHS lymphoblasts with MLCL AT-1 expression construct increased CL, improved mitochondrial basal respiration and protein leak, and decreased the proportion of cells producing superoxide but did not restore CL molecular species composition to control levels. In addition, BTHS lymphoblasts exhibited higher rates of glycosis compared with healthy controls to compensate for reduced mitochondrial respiratory function. Mitochondrial supercomplex assembly was significantly impaired in BTHS lymphoblasts, and transfection of BTHS lymphoblasts with MLCL AT-1 expression construct did not restore supercomplex assembly. The results suggest that expression of MLCL AT-1 depends on functional TAZ in healthy cells. In addition, transfection of BTHS lymphoblasts with an MLCL AT-1 expression construct compensates, but not completely, for loss of mitochondrial respiratory function.

CL₃ is a major polyglycerophospholipid found exclusively in mitochondria, predominantly in the inner mitochondrial membrane (IMM) (1–3). CL plays key roles in mitochondrial protein transport (4) and mitochondrially mediated apoptosis (5–8), acts as the “glue” that holds mitochondrial supercomplexes (SCs) together (9–12), is involved in the signaling pathway that exists between mitochondria and vacuoles (13), and is required for mitophagy (14). CL is composed of a polar headgroup, a glycerol backbone, and four acyl chains (15). CL is synthesized de novo through the CDP-diacylglycerol pathway (for a review, see Ref. 16). Subsequent to its de novo biosynthesis, it is these four fatty acyl chains that must be remodeled with specific fatty acids to ensure proper CL function (for a review, see Ref. 17). The principal gene involved in CL remodeling is tafazzin (TAZ). TAZ is responsible for the production of the protein TAZ, a transacylase located in mitochondria that transfers acyl chains from phospholipids such as phosphatidylcholine and phosphatidylethanolamine to monolysocardiolipin (MLCL) to produce CL (18). This transfer of acyl chains between CL and other phospholipids is required to ensure that specific CL species are produced (19). The acyl specificity of the TAZ reaction may result from either the enzyme itself or the physical properties of lipids (20, 21).

Barth syndrome (BTHS) is a rare X-linked recessive disease first characterized by Dr. Peter Barth and later by Dr. Richard Kelley that results in various cardiomyopathies, neutropenia, skeletal myopathies, and 3-methylglutaconic aciduria (22, 23).

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The abbreviations used are: CL, cardiolipin; TAZ, tafazzin; BTHS, Barth syndrome; MLCL, monolysocardiolipin; AT, acyltransferase; IMM, inner mitochondrial membrane; SC, supercomplex; ROS, reactive oxygen species; αFP, α subunit of the mitochondrial trifunctional protein; BN, blue native; CI, complex I; OCR, oxygen consumption rate; SRC, spare respiratory capacity; ECAR, extracellular acidification rate; CI, complex II; CII, complex III; CIV, complex IV; CV, complex V; A/A, antibiotic/antimycotic; BisTris, 2-[bis(2-hydroxyethyl)aminio]-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1-bis(hydroxymethyl)ethyl]glycine; VDAC, voltage-dependent anion channel; HADHA, hydroxacyl-CoA dehydrogenase-3-ketoacyl-CoA thiolase/enough-CoA hydratase (trifunctional protein) alpha subunit.
It is the only known disease exclusively associated with dysfunctional CL remodeling (24). BTHS is caused by various mutations in the TAZ gene that result in reduced CL (for a review, see Ref. 25). Skeletal muscle mitochondria from BTHS patients exhibit mitochondrial respiratory chain disturbances. In addition, BTHS cells exhibit mitochondrial fragmentation (26), impaired mitochondrial function (11, 27), SC disassembly (28), and increased reactive oxygen species (ROS) production (29). It is unclear why specific CL species are predominant in tissues such as the heart and skeletal muscle. What is clear is that disruption of TAZ (and therefore CL remodeling) leads to development of BTHS.

Sclame and Rüstow (30) initially identified an acyl-CoA–dependent mechanism of CL remodeling in rat liver mitochondria. In that study, a cycle involving CL deacylation by phospholipase A2 followed by MLCL reacylation using linoleoyl-CoA as substrate was observed. In vitro MLCL acyltransferase (AT) activity was initially demonstrated in crude rat heart mitochondria and later shown to be localized to the inner leaflet of the IMM (31). The enzyme was subsequently purified from pig liver mitochondria (32). It is a 59-kDa splice variant of the 74-kDa α subunit of the mitochondrial trifunctional protein (αTFP) encoded by the HADHA gene (33). Peptide sequence analysis revealed a match with a then unknown 59-kDa human protein (protein accession number AAX93141). Alignment of the human αTFP and MLCL AT-1 protein sequences revealed that they were identical except for the first 227 amino acids, which are absent in the MLCL AT-1 protein sequence. Despite the identification and demonstration of an in vitro activity for MLCL AT-1, the role that this protein plays in mitochondrial respiratory function is largely unknown. In this study, we examined how TAZ influences MLCL AT-1 expression in healthy and BTHS lymphoblasts and how expression of an MLCL AT-1 expression construct influences mitochondrial respiratory function in BTHS lymphoblasts.

Results

Transfection of BTHS cells with MLCL AT-1 expression construct increases CL

Initially we examined CL levels in age-matched healthy (3798) lymphoblasts, BTHS (618) lymphoblasts, 3798 cells transfected with TAZ RNAi, 618 cells transfected with a MLCL AT-1 expression construct, and 3798 cells cotransfected with TAZ RNAi and an MLCL AT-1 expression construct (3798 TAZ RNAi + MLCL AT-1), 618 cells (618 Mock), and 618 cells transfected with MLCL AT-1 expression construct (618 MLCL AT-1). Data represent mean ± S.D., n = 4. ns, not significant, *, p < 0.05 compared with 3798 control; **, p < 0.01 compared with 3798 control; #, p < 0.001 compared with 618 mock. B, MLCL AT-1 enzyme activity in the above cells. Data represent mean ± S.D., n = 3. *, p < 0.05 compared with 3798 control; **, p < 0.001 compared with 3798 control; #, p < 0.001 compared with 618 mock. Error bars represent S.D.

Reduction in TAZ increases MLCL AT-1 mRNA, protein expression, and enzyme activity in healthy lymphoblasts but not in BTHS lymphoblasts

We next examined MLCL AT-1 enzyme activity in the above cells. 3798 cells transfected with TAZ RNAi exhibited a 1.5-fold increase (p < 0.01) in MLCL AT-1 enzyme activity compared with 3798 control cells (Fig. 1B). Cotransfection of 3798 cells with TAZ RNAi and MLCL AT-1 expression construct resulted in a 2.5-fold increase (p < 0.01) in MLCL AT-1 enzyme activity compared with 3798 control cells. MLCL AT-1 enzyme activity was 22% lower (p < 0.05) in 618 cells compared with 3798 cells. Transfection of MLCL AT-1 expression construct in 618 cells resulted in an approximate 2-fold increase (p < 0.001) in MLCL AT-1 enzyme activity compared with mock-transfected 618 cells (Fig. 1B). Thus, a reduction in TAZ increases compensatory MLCL AT-1 enzyme activity in healthy cells but not in BTHS cells. In addition, transfection of MLCL AT-1 expression construct in either 3798 or 618 cells increases MLCL AT-1 enzyme activity.

We next examined the relative mRNA expression of both TAZ and MLCL AT-1 in age-matched healthy (3798) and BTHS (618) lymphoblasts and in 3798 cells transfected with TAZ RNAi to determine how reduction in TAZ influences MLCL AT-1 mRNA expression. Transfection of 3798 cells with TAZ RNAi resulted in a 45% decrease in TAZ mRNA expression (p < 0.001; Fig. 2A) and a 2-fold increase in MLCL AT-1 mRNA expression (p < 0.05; Fig. 2B) compared with 3798 control cells. TAZ mRNA expression was reduced 50% in 618 cells compared with 3798 control cells.
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Table 1

| Molecular species          | 3798 control | 3798 TAZ RNAi | 3798 mock | 618 Mock | 618 MLCL AT-1 |
|----------------------------|-------------|--------------|-----------|---------|--------------|
| CL (18:2)(18:1)            | 2.1 ± 0.3   | 2.1 ± 0.3    | 2.6 ± 0.3 | 0.0 ± 0.0| 0.0 ± 0.0    |
| CL (18:1)(18:2)            | 1.3 ± 0.6   | 0.9 ± 0.2    | 0.3 ± 0.5 | 0.3 ± 0.5| 0.3 ± 0.5    |
| CL (18:2)(18:1)            | 8.4 ± 0.7   | 11.4 ± 0.9   | 9.1 ± 2.0 | 50.5 ± 45| 50.5 ± 45    |
| CL (18:2)(18:1)(20:3)      | 3.7 ± 0.9   | 5.1 ± 0.6    | 2.8 ± 2.5 | 1.1 ± 1.9| 1.1 ± 1.9    |
| CL (18:2)(18:1)(22:6)      | 1.1 ± 0.4   | 0.4 ± 0.4    | 0.5 ± 0.6 | 0.3 ± 0.5| 0.3 ± 0.5    |
| CL (18:1)(18:2)(22:6)      | 1.8 ± 0.8   | 1.5 ± 0.4    | 0.6 ± 0.6 | 0.4 ± 0.7| 0.4 ± 0.7    |
| CL (18:2)(16:1)            | 9.8 ± 0.4   | 9.9 ± 1.8    | 12.8 ± 5.7| 3.9 ± 6.8| 3.9 ± 6.8    |
| CL (18:2)(20:3)            | 6.0 ± 0.8   | 6.1 ± 0.8    | 7.0 ± 2.0 | 2.3 ± 4.0| 2.3 ± 4.0    |
| CL (18:2)(20:4)            | 1.1 ± 0.1   | 1.4 ± 0.3    | 2.2 ± 0.5 | 0.6 ± 1.1| 0.6 ± 1.1    |
| CL (18:2)(16:1)            | 5.4 ± 1.6   | 5.2 ± 2.1    | 4.8 ± 0.5 | 1.6 ± 2.8| 1.6 ± 2.8    |
| CL (18:2)(16:1)            | 1.9 ± 0.9   | 1.4 ± 1.0    | 0.0 ± 0.0 | 12.2 ± 19.3| 12.2 ± 19.3 |
| CL (18:2)(16:1)            | 14.5 ± 0.7 | 13.1 ± 2.5   | 7.0 ± 0.7 | 3.1 ± 5.3| 3.1 ± 5.3    |
| CL (18:2)(18:1)            | 14.5 ± 0.7 | 22.4 ± 1.4   | 19.7 ± 4.2| 6.4 ± 11.1| 6.4 ± 11.1   |
| CL (18:2) (18:2)           | 7.6 ± 1.6   | 15.8 ± 2.5   | 25.2 ± 6.8| 16.8 ± 14.5| 16.8 ± 14.5 |
| CL (18:2) (18:2)           | 1.0 ± 0.6   | 1.1 ± 0.4    | 3.0 ± 0.8 | 0.5 ± 0.8| 0.5 ± 0.8    |

with 3798 cells ($p < 0.01$; Fig. 2C). Surprisingly, MLCL AT-1 mRNA expression was 45% lower in 618 cells compared with 3798 cells ($p < 0.001$; Fig. 2D). Transfection of 618 cells with MLCL AT-1 expression construct resulted in a 2.5-fold increase in MLCL AT-1 mRNA expression compared with mock-transfected 618 cells ($p < 0.01$; Fig. 2E). Thus, a reduction in TAZ increased MLCL AT-1 mRNA expression in healthy cells but not in BTHS cells.

We next examined the protein expression of TAZ and MLCL AT-1 in these cells. Transfection of 3798 cells with TAZ RNAi resulted in a 40% decrease ($p < 0.001$) in TAZ protein expression compared with 3798 control cells (Fig. 3, A and B). As anticipated, 618 cells exhibited lower levels of detectable TAZ protein, a 90% decrease ($p < 0.001$) compared with 3798 cells. Transfection of 3798 cells with TAZ RNAi resulted in a 1.5-fold increase ($p < 0.001$) in MLCL AT-1 protein expression compared with 3798 control cells (Fig. 3, C and D). MLCL AT-1 protein expression was 75% lower ($p < 0.001$) in 618 cells compared with 3798 cells. Transfection of 618 cells with increasing amounts of MLCL AT-1 expression construct resulted in an increasing amount of MLCL AT-1 protein expression in these cells (Fig. 2, E and F). A 6-fold ($p < 0.001$) increase in MLCL AT-1 protein expression was observed in 618 cells transfected with 20 μg of MLCL AT-1 expression construct compared with mock-transfected 618 cells (Fig. 3, E and F). Addition of increasing amounts of MLCL AT-1 expression construct in 618 cells did not result in significant alteration in the level of aTTP protein expression compared with mock-transfected 618 cells (Fig. 3, E and G). Thus, transfection with TAZ RNAi increases MLCL AT-1 protein expression in healthy cells but not in BTHS cells. In addition, transfection of BTHS cells with MLCL AT-1 expression construct results in an increase in MLCL AT-1 protein expression in these cells.

Transfection of BTHS cells with MLCL AT-1 expression construct does not affect mitochondrial supercomplex assembly

We performed BN PAGE of mitochondrial fractions prepared from 3798 cells, 3798 cells transfected with TAZ RNAi, 618 cells, and 618 cells transfected with MLCL AT-1 expression construct to examine mitochondrial SC protein assembly. Transfection of 3798 cells with TAZ RNAi did not appear to affect mitochondrial SC protein assembly compared with 3798 cells (Fig. 4A, lanes 1 and 2). In contrast, higher order SCs were absent in 618 cells compared with 3798 cells (Fig. 4A, lanes 1 and 3). Transfection of 618 cells with MLCL AT-1 expression construct did not affect mitochondrial SC protein assembly compared with mock-transfected 618 cells (Fig. 4A, lanes 3 and 4).

Complex I (CI) in-gel enzyme activity assays were then performed to probe for CI enzyme activity and CI enzyme activity within the SCs of these cells. Fig. 4B shows the presence of CI as well as CI enzyme activity–containing SCs at higher molecular weights. Transfection of 3798 cells with TAZ RNAi resulted in a significant decrease in enzyme activity of several CI-containing SCs compared with 3798 control cells (Fig. 4, B, lanes 1 and 2, and C). In 618 cells, a significant reduction in enzyme activity of several CI-containing SCs was observed compared with 3798 cells but not CI (lanes 4, B, lanes 3, 4, and C). Transfection of 618 cells with MLCL AT-1 expression construct did not affect the enzyme activity of CI or the majority of CI-containing SCs compared with 618 mock-transfected cells (Fig. 4, B, lanes 3 and 4, and C). Thus, transfection of BTHS lymphoblasts with MLCL AT-1 expression construct does not affect mitochondrial SC assembly.

Transfection of BTHS cells with MLCL AT-1 expression construct improves mitochondrial basal respiration and proton leak but not spare respiratory capacity or ATP coupling efficiency

We next examined mitochondrial respiration in these cells. Basal respiration, measured as oxygen consumption rate (OCR) (pmol/min/100,000 cells) before the addition of oligomycin, was ~4-fold higher ($p < 0.001$) in 618 cells compared with 3798 cells (Fig. 5, A and B). Transfection of 3798 cells with TAZ RNAi resulted in an approximate 3-fold increase ($p < 0.001$) in basal respiration compared with 3798 control cells. Transfection of 618 cells with MLCL AT-1 expression construct reduced basal respiration to near that of mock-transfected 618 cells. The reduction in basal respiration in these cells mimicked that observed in control 3798 cells. However, basal respiration was still significantly higher ($p < 0.001$) in 618 cells transfected with MLCL AT-1 expression construct compared with 3798 control cells.

Proton leak was ~9-fold higher ($p < 0.01$) in 618 cells compared with 3798 cells (Fig. 5, A and C). Transfection of 3798 cells with TAZ RNAi resulted in a 5-fold increase ($p < 0.001$) in proton leak compared with 3798 control cells. Transfection of 618 cells with MLCL AT-1 expression construct decreased proton leak 70% compared with mock-transfected 618 cells. The reduction in proton leak in these cells mimicked that observed in control 3798 cells. However, proton leak was still 2.8-fold higher ($p < 0.001$) in 618 cells transfected with MLCL AT-1 expression construct compared with control 3798 cells. In 3798 lymphoblasts, a spare respiratory capacity (SRC) of 50% was observed, whereas 618 lymphoblasts exhibited an SRC of 30% (Fig. 5, A and D). Thus, SRC was decreased 40% ($p <
In 3798 cells, ~70% of mitochondrial respiration was coupled to ATP production (Fig. 5, A and E). Transfection of 3798 cells with TAZ RNAi resulted in a 10% decrease ($p < 0.001$) in ATP coupling efficiency compared with 3798 control cells. ATP coupling efficiency was reduced to 35% ($p < 0.001$) in 3798 cells cotransfected with TAZ RNAi and MLCL AT-1 expression construct (MLCL AT-1 with TAZ RNAi). *$p < 0.05$ compared with MLCL AT-1 control. C, TAZ mRNA expression in 3798 (3798 TAZ) and 618 (618 TAZ) cells. ** $p < 0.01$ compared with 3798 TAZ. D, MLCL AT-1 mRNA expression in 3798 (3798 MLCL AT-1) and 618 (618 MLCL AT-1) cells. *** $p < 0.001$ compared with 3798 MLCL AT-1. E, MLCL AT-1 mRNA expression in 618 (618 Mock) and 618 cells transfected with MLCL AT-1 expression construct (618 MLCL AT-1). ** $p < 0.01$ compared with 618 mock. Error bars represent S.D.

We next examined glycolytic activity to further assess how energy production may be affected in 3798 and 618 cells. Extracellular acidification rates (ECARs) were measured in 3798 and 618 cells after addition of glucose. ECAR was increased 50% ($p < 0.001$) in 618 cells compared with 3798 cells (Fig. 5F). Thus, BTHS lymphoblasts exhibit an increase in glycolysis.

**Transfection of BTHS cells with MLCL AT-1 expression construct attenuates ROS production**

We examined superoxide production in 3798 cells, 618 cells, and 618 cells transfected with MLCL AT-1 expression construct. Cells were stained with MitoSOX (an indicator of superoxide production), and flow cytometry analysis was performed (Fig. 6). The right peak indicates the proportion of cells positive for MitoSOX staining, whereas the left peak indicates the proportion of cells that are not positive for MitoSOX staining. The
proportion of cells positive for superoxide production was significantly higher \((p < 0.01)\) in 618 mock-transfected cells compared with 3798 control cells, \(-75\) versus \(50\%)\), respectively (Fig. 6, A and D). Transfection of 618 cells with MLCL AT-1 expression construct resulted in a significant reduction \((p < 0.05)\), from \(-75\) to \(60\%)\), in the proportion of cells positive for superoxide production compared with mock-transfected 618 cells (Fig. 6, B and D). As a positive control, addition of antimycin A
(an inducer of superoxide production) resulted in 100% of all cells positive for superoxide production with no significant differences between 3798 and 618 cells (Fig. 6C). Thus, transfection of BTHS lymphoblasts with MLCL AT-1 expression construct attenuates ROS production.

Discussion

It is now well documented that TAZ is the major CL-remodeling enzyme in mammalian cells (18). We had previously observed that MLCL AT-1 functions by remodeling CL with specific acyl chains (31–33, 37). The objective of this study was to determine what relationship, if any, exists between TAZ and MLCL AT-1 with regard to CL remodeling and whether expression of an MLCL AT-1 construct in BTHS lymphoblasts improves mitochondrial respiratory function. We utilized two models of lowered TAZ functional expression, transfection of healthy 3798 cells with TAZ RNAi and transfection of 618 BTHS lymphoblasts, which exhibit a mutation in TAZ.

Our results demonstrate that CL content was significantly lower in 618 BTHS lymphoblasts compared with 3798 controls. In contrast, the molecular species composition of CL was only marginally altered in these cells. Transfection of healthy 3798 cells with TAZ RNAi resulted in significant elevation in MLCL AT-1 gene, protein, and enzyme activity, whereas the CL level and its molecular species composition remained largely unchanged. In addition, transfection of BTHS lymphoblasts with MLCL AT-1 expression construct increased the CL level but did not alter its molecular species composition. These results indicate that a reduction in TAZ in healthy 3798 cells leads to a compensatory increase in MLCL AT-1 expression.
Figure 5. Transfection of BTHS cells with MLCL AT-1 expression construct improves basal respiration and attenuates proton leak. A, oxygen consumption rates in 3798 cells (3798 Control), 3798 cells transfected with TAZ RNAi (3798 TAZ RNAi), 618 cells (618 Mock), and 618 cells transfected with MLCL AT-1 expression construct (618 MLCL AT-1). Oligo, oligomycin; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; AA, antimycin. Basal respiration (B), proton leak (C), spare respiratory capacity (D), and ATP coupling (E) from the above cells are shown. Data represent mean ± S.D., n = 3. #, p < 0.001; †, p < 0.01 compared with 3798 control; ‡, p < 0.001 compared with 618 mock; ns, not significant. F, ECARs in 3798 and 618 cells after addition of glucose. Data represent mean ± S.D., n = 3. ***, p < 0.001 compared with 3798. Error bars represent S.D.
that may prevent complete CL loss. Thus, in healthy cells, the role of MLCL AT-1 may be to attenuate pathological decreases in CL. It was previously hypothesized that MLCL AT-1 may compensate for loss of CL (37). In that study, 2-deoxyglucose induction of phospholipase A2 in H9c2 cardiac cells did not result in an alteration in CL pool size or its fatty acyl molecular species composition but did increase MLCL AT enzyme activity. Thus, MLCL AT-1 may play a role in maintaining CL homeostasis and mitochondrial function in healthy cells. However, other factors may be involved in the attenuation of CL reduction in 3798 cells transfected with TAZ RNAi. CL has among the slowest turnover rates compared with other phospholipids. A previous study reported that CL exhibited a half-life of 10.4 days compared with other phospholipids, which experience faster turnover rates (39). The healthy 3798 lymphoblasts used in our study were transfected with TAZ RNAi and subsequently incubated for 48 h. Thus, although this procedure resulted in a substantial reduction in TAZ gene expression, it is likely that the slow turnover rate of CL and its inability to undergo remodeling were responsible for the lack of reduction in CL and alteration in molecular species composition.

618 BTHS lymphoblasts exhibited TAZ protein and CL levels that were significantly lower compared with healthy 3798 controls. In 618 BTHS lymphoblasts, we did not observe an increase in MLCL AT-1 expression. In fact, MLCL AT-1 mRNA expression was significantly lower in 618 cells compared with healthy 3798 controls. This was surprising considering the results obtained in healthy 3798 lymphoblasts that indicated a potential compensatory role for MLCL AT-1 when TAZ levels were reduced by transfection with TAZ RNAi. BTHS lymphoblast mitochondria exhibit a variety of structural abnormalities

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**Figure 6. Transfection of BTHS cells with MLCL AT-1 expression construct attenuates ROS production.** 3798 cells (3798 Control), 618 cells (618 Mock), and 618 cells transfected with MLCL AT-1 expression construct (618 MLCL AT-1) were incubated with MitoSOX, and the proportion of cells positive for superoxide production was determined. The y axis in A, B, and C represents mean fluorescence intensity. A, superoxide production in 3798 cells (3798 Control) and 618 cells (618 Mock). B, superoxide production in 618 cells (618 Mock) and 618 cells transfected with MLCL AT-1 expression construct (618 MLCL AT-1). C, addition of antimycin A as a control, resulting in nearly 100% of cells being positive for superoxide production. Representative plots are depicted. D, distribution of cells positive for superoxide production. Data represent mean ± S.D., n = 3. **, p < 0.01 compared with 3798 control; *, p < 0.05 compared with 618 mock; ns, not significant compared with 3798 control. Error bars represent S.D.
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including decreased cristal surface area, increased mitochondrial volume, and fragmented mitochondria (6, 40). It is possible that the extensive mitochondrial damage in BTHS cells prevents them from utilizing MLCL AT-1 on the IMM to compensate for decreases in TAZ. Thus, in BTHS cells with grossly impaired mitochondria, endogenous MLCL AT-1 cannot maintain normal levels of CL.

One of the main proposed roles of CL is to act as the glue that holds mitochondrial SCs together (9, 10, 28, 41, 42). Previous studies have shown that TAZ deficiency in yeast or BTHS lymphoblasts resulted in destabilized supercomplexes (28, 43). Because 618 BTHS lymphoblasts exhibited significantly lower levels of TAZ and CL, we examined the effect of increasing MLCL AT-1 protein on mitochondrial respiratory function in these cells. We chose to examine CI SC formation because, unlike other complexes of the electron transport chain, CI predominantly exists in SCs rather than as an individual unit (44). 618 BTHS cells exhibited a pronounced alteration in CI-containing SC assembly compared with healthy 3798 cells or healthy 3798 cells transfected with TAZ RNAi. This is likely due to the complete absence of functional TAZ protein. Interestingly, transfection of 618 BTHS cells with MLCL AT-1 expression construct did not restore CI-containing SC assembly even though CL levels were elevated. This observation, coupled with the observation that the level of CL and its molecular species composition were essentially unaltered in healthy 3798 cells transfected with TAZ RNAi, suggests that CL alone is not required for SC assembly in these cells. Because transfection of 618 BTHS cells with MLCL AT-1 expression construct did not restore CL levels to that observed in healthy cells, it is possible that this may have prevented restoration of CI-containing SC assembly in BTHS cells. Alternatively, the lack of restoration of CI-containing SC assembly in BTHS cells may be due to the observation that expression of the MLCL AT-1 construct in these cells did not restore the molecular species composition of CL to that of healthy cells.

In BTHS cells, basal respiration was significantly elevated compared with healthy controls. In addition, healthy 3798 lymphoblasts transfected with TAZ RNAi exhibited significantly higher basal respiration rates compared with controls. Basal respiration, defined as OCR prior to the addition of oligomycin, is controlled by cellular ATP turnover as well as proton leak and thus changes in response to ATP demand (45). Thus, a reduction in TAZ results in impaired ATP production, which forces cells to increase their OCRs. Transfection of MLCL AT-1 expression construct in 618 BTHS cells resulted in a significant decrease in basal respiration and restored OCRs to near that of healthy 3798 lymphoblasts. These data indicate that transfection of BTHS cells with MLCL AT-1 expression construct improved basal respiration.

Proton leak, measured after the addition of oligomycin, was higher in 618 BTHS cells compared with healthy 3798 cells or 3798 cells transfected with TAZ RNAi. A high proton leak is indicative of mitochondrial membrane damage (45). This observation, coupled with our BN PAGE results demonstrating impaired SC assembly, indicates that the IMM of BTHS cells or 3798 cells transfected with TAZ RNAi is significantly damaged compared with healthy cells. However, the degree of proton leak in healthy 3798 cells transfected with TAZ RNAi was lower than that in 618 cells. This was likely due to the lack of CL loss in the healthy 3798 cells transfected with TAZ RNAi. It is possible that the observed increase in MLCL AT-1 expression in healthy 3798 cells transfected with TAZ RNAi was responsible for maintaining CL levels in these cells. This is supported by our observation in which transfection of 618 BTHS cells with MLCL AT-1 expression construct increased CL and significantly lowered proton leak. The reduction in proton leak explains the improvement in basal respiration in these cells. These data indicate that transfection of BTHS cells with MLCL AT-1 expression construct attenuates proton leak.

A healthy spare respiratory response is an important physiological process, particularly for cells that experience variable ATP demand (45). Decreased SRC has been implicated in heart disease and cell death in smooth muscle cells (45, 46). 618 BTHS cells exhibited significantly lower SRC compared with healthy 3798 cells. Thus, BTHS cells are less able to respond to the demands for increased workloads under situations of stress. Interestingly, healthy 3798 lymphoblasts transfected with TAZ RNAi exhibited a significant increase in SRC. This observation suggests that healthy lymphoblasts are better able to respond to the stress induced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone. Thus, despite decreased TAZ in these cells, SRC was not impaired. Transfection of 618 BTHS cells with MLCL AT-1 expression construct did not increase SRC, indicating that, similar to 618 BTHS cells, these cells are less able to respond to the demands for increased workloads under situations of stress.

BTHS cells and healthy 3798 lymphoblasts transfected with TAZ RNAi exhibited a decrease in ATP coupling efficiency. Transfection of 618 BTHS cells with MLCL AT-1 expression construct did not improve ATP coupling efficiency. These data indicate that the small increase in CL in 618 cells mediated by transfection with MLCL AT-1 expression construct may have a more prominent effect on mitochondrial membranes than on complexes of the electron transport chain. CL synthase mutant Drosophila and lymphoblasts from BTHS patients, with decreased CL, exhibited reduced complex V (CV) and destabilization of supramolecular structures (6, 47). CV is required for ATP production and has been shown to rely on CL for its proper function. If CV function is impaired in BTHS cells, then this might explain the observed decreased ATP coupling efficiency. This is supported by our BN PAGE results in which, despite the modest increase in CL in 618 BTHS cells transfected with MLCL AT-1 expression construct, CV remained lower than in 3798 cells, and SC formation remained impaired. Interestingly, 618 BTHS lymphoblasts exhibited an increase in ECAR upon glucose addition compared with 3798 healthy cells, indicating that these cells exhibit an increase in glycolysis. If this were the case in other tissues, then this might further explain the increase in glucose disposal rates observed in BTHS patients (48).

Disruption of SCs is known to result in increased production of ROS (49, 50). Previous studies have shown that elevated production of ROS results in mitochondrial dysfunction mediated by CL peroxidation (51, 52). In addition, cell models of BTHS indicate that BTHS patients may exhibit elevated ROS produc-
tion (11, 27). We observed a greater proportion of 618 BTHS lymphoblasts producing superoxide compared with 3798 healthy cells. This correlated with our BN PAGE results that showed decreased SC formation. Transfection of 618 BTHS cells with MLCL AT-1 expression construct resulted in a lower proportion of cells producing superoxide compared with mock-transfected BTHS cells. However, if SCs prevent aberrant production of ROS, then why was the observed decrease in ROS in 618 BTHS cells transfected with MLCL AT-1 expression construct not accompanied by an increase in SC formation? As previously discussed, BTHS lymphoblasts exhibited a significantly higher proton leak compared with healthy cells. An increase in ROS leads to increased proton leak (for a review, see Ref. 53). Therefore, the decreased proportion of BTHS cells transfected with MLCL AT-1 expression construct that were positive for superoxide production indicate that a decrease in ROS production may be responsible, at least in part, for the decreased proton leak observed. However, this still does not explain the reduced SC assembly observed in these cells. The lack of SC assembly restoration in 618 cells transfected with MLCL AT-1 expression construct may be attributed to the limitation of that particular experiment. BN PAGE allows for the detection of proteins using non-denaturing conditions, which help preserve protein-to-protein interactions, and is therefore useful for the study of SCs. However, despite the mild conditions implemented by BN PAGE, some protein denaturation still occurs. Clear native PAGE, a technique that is milder than BN PAGE, has also been used to study SCs (54). A previous study using a CL synthase mutant yeast strain showed that CL levels were significantly reduced compared with controls (9). This was accompanied by dissociation of the CIIIA/CIV SCs as detected by BN PAGE. However, using clear native PAGE, the authors observed that SC formation was not entirely absent in these CL-deficient yeast strains. This observation led to their conclusion that CL may be needed for SC stabilization but not formation. Thus, it is possible that SC formation may not be entirely absent in 618 BTHS lymphoblasts, but the drastic reduction in CL, or accumulation of MLCL, may lead to increased SC dissociation. The increase in CL in BTHS cells transfected with MLCL AT-1 expression construct may not be sufficient to stabilize these SCs. However, it may be sufficient to improve SC function enough to result in decreased ROS production.

In summary, our results demonstrate that MLCL AT-1 expression is elevated in response to reduced TAZ expression in healthy lymphoblasts but not in BTHS lymphoblasts. In addition, 618 BTHS lymphoblasts exhibit SC destabilization, mitochondrial respiratory dysfunction, elevated production of ROS, and increased glycolysis, and transfection of these cells with MLCL AT-1 expression construct results in an improvement to basal mitochondrial respiration and proton leak and a reduction in the proportion of cells positive for superoxide production.

Experimental procedures

Materials

Epstein–Barr virus–transformed lymphoblasts (identifier 3798) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Age-matched BTHS lymphoblasts (patient 618; Exon 2, c. 171 del. A (frameshift)) were generously donated by Dr. Richard Kelly (The John Hopkins University). RPMI 1640 medium, Opti-MEM, Stealth RNAi, and other cell culture reagents and supplements were ordered from Life Technologies. TAZ RNAi sequences were as follows: control, 5’-CCUGAUGCUUUUCGCCAACAGAGUA-3’ (sense) and 5’-UACUGUGUUGCAGGGAGUACAGG-3’ (antisense); tafazzin, 5’-CCUACAGCUGUUCCGGAAGCAGACGAG-3’ (sense) and 5’-UACUGUUGGCAGCAGCGUGAAGG-3’ (antisense). RNAProtec® reagent, RNeasy® Plus Mini kit, Qiagen blood Redder homogenizer columns, QuantiTect® Probe RT-PCR kit, and other RT-PCR components were obtained from Qiagen (Cambridge, MA). The following primers used for RT-PCR detection were ordered from Life Technologies: tafazzin: forward, 5’-GCAGACATCGCTTCCACCA-3’; reverse, 5’-TCTGGTAGACGGCCATCTCT-3’; MLCL AT-1: forward, 5’-GAAGTCATCCGAATCCTCCA-3’; reverse, 5’-TTTCGCTACATCCACACCA-3’; 18S: forward, 5’-AACCGCTTACACATCCAAG-3’; reverse, 5’-CCTGCCATGGTTCCTGTA-3’. Tafazzin (catalog number ab105104), HADHA (catalog number ab137663), and voltage-dependent anion channel 1 (VDAC1) (catalog number ab15895) primary antibodies used for Western blot analysis were obtained from Abcam (Cambridge, MA), and goat anti-rabbit IgG-HRP (catalog number sc-2004) was from Santa Cruz Biotechnology Inc. (Dallas, TX). Other Western blotting components were obtained from Bio-Rad and GE Healthcare. Radiolabels were obtained from PerkinElmer Life Sciences. Ecolite scintillant was obtained from ICN Biochemicals (Montreal, Quebec, Canada). Lipid standards were obtained from Serdary Research Laboratories (Englewood Cliffs, NJ). Thin-layer chromatographic plates (silica gel G). Thin-layer chromatographic plates (silica gel G, 0.25-mm thickness) were obtained from Merck. BN PAGE components and reagents were obtained from Life Technologies and Sigma-Aldrich. Mitochondrial stress test and glycolytic analysis components were obtained from Seahorse Bioscience (North Billerica, MA). Cell-Tak was obtained from Corning. MitoSOX and Amplex UltraRed molecular probes were obtained from Life Technologies. All other biochemical agents, components, and drugs were American Chemical Society (ASC) grade and obtained from either Sigma-Aldrich or Thermo Fisher Scientific (Carlsbad, CA).

MLCL AT-1 plasmid preparation

The MLCL AT-1 plasmid was prepared as described by Taylor and Hatch (33). Briefly, the primers for the MLCL AT-1 protein were amplified using 1 µg of HeLa cell RNA. The full-length cDNA sequences were incorporated into pcDNA 3.1(+) using the TOPO cloning reaction. Once the plasmid was constructed, it was transformed into Escherichia coli One Shot® bacteria via a chemical reaction using super optimal broth with catabolite repression (S.O.C.) medium followed by inoculation into ampicillin-containing agar and allowed to grow overnight. The next day, colonies were isolated and inoculated into 5 ml of ampicillin-containing LB medium and cultured at 37 °C in an orbital shaker set at 200 rpm overnight. Plasmid purification from E. coli was conducted using a Qiagen Plasmid Midi kit (catalog number 12143) as described by the manufacturer's
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Protocol. DNA sequencing (Manitoba Institute of Cell Biology) was used to verify the plasmid sequences.

Cell culture and transfection

The cell lines were grown in RPMI 1640 medium containing 10% FBS and 1% antibiotic/antimycotic (A/A) solution at 37 °C and 5% CO₂ in a Thermo Scientific Sercycle CO₂ incubator (HEPA Class 200). Cell medium was changed every 48 h, and the cells were passaged every 5 days. Lymphoblasts were transfected via electroporation with a BTX Electroporation System Manipulator 600 at 250 V for 15–20 ms. To 5 × 10⁶ cells, 2 nmol of Stealth RNAi (Invitrogen) were added. To knock down TAZ, 3798 cells were transfected with TAZ RNAi sequence (scrambled sequence was used as a negative control). In other experiments, 618 cells were transfected with either 20 μg of a MLCL AT-1–containing plasmid or 20 μg of an empty vector as above. In some experiments, cells were transfected with 0–80 μg MLCL AT-1–containing plasmid. After transfection, cells were incubated in 5 ml of RPMI 1640 medium without the addition of FBS or A/A solution for 6 h at 37 °C and 5% CO₂. After 6 h, 15 ml of RPMI 1640 medium with 10% FBS and 1% A/A solution were added to each sample and incubated at 37 °C and 5% CO₂ for an additional 42 h.

RNA isolation and RT-PCR

RNA was isolated from healthy and BTHS lymphoblasts using RNAprotect reagent, RNaseasy Plus Mini kit, and Qiashredder homogenizer columns as described by the manufacturer’s protocol. One-step RT-PCR was performed using the QuantiTect Probe RT-PCR kit, and the dsDNA stain SYBR Green was used as indicated by the manufacturer. Final primer concentration was 0.4 μM and final RNA template concentration was 8.0 ng/μl in a reaction mixture volume of 25 μl. Relative gene expression analysis was performed using a Master Cycler ep Realplex system. Relative gene expression was calculated using the 2^−ΔΔCt method as described previously (55).

Immunoblotting (SDS-PAGE)

Mitochondrial protein from healthy and BTHS lymphoblasts was used for all Western blotting. A mitochondrial isolation kit from Abcam (catalog number ab110170) was utilized to isolate mitochondrial fractions as described in the kit’s protocol. Mitochondrial protein concentrations were determined using the Bradford method (56). To 15 μg of mitochondrial protein, an equal volume of 2X Laemmli buffer (Bio-Rad, catalog number 1610737) supplemented with 5% 2-mercaptoethanol was added and mixed by pipetting. The samples were boiled at 95 °C for 5 min and then allowed to cool before centrifugation at 2000 rpm for 1 min. The entire volume was then loaded onto individual wells of a 4–15% polyacrylamide precast gel (Bio-Rad, catalog number 4561086), and electrophoresis was performed at 100 V for 1 h. This was followed by a wet transfer of proteins onto a polyvinylidene difluoride (PVDF) membrane performed at 100 V for 1 h at 4 °C. The membrane was then blocked for 1 h at room temperature using 5% Amersham ECL Prime blocking reagent (GE Healthcare, catalog number RP418) in a solution of TBS containing 0.5% Tween 20 (TBS-T). The membrane was then washed three times at 5-min intervals with TBS-T before an overnight incubation (4 °C) with the respective primary antibody in a blocking buffer solution of 3% BSA in TBS-T. The next day, the primary antibody solution was removed, and the membrane was washed three times at 5-min intervals with TBS-T (room temperature). The membrane was then incubated in a secondary antibody blocking solution of 3% BSA in TBS-T for 1 h at room temperature. The secondary antibody solution was then removed, and the membrane was washed with TBS-T three times at 10-min intervals. To develop the membrane for protein band visualization, ECL blocking agent (GE Healthcare, catalog number RPN2125) was used as specified by the manufacturer’s protocol. A ChemiDoc™ MP imaging system was used to visualize protein bands and capture images, and the relative band intensities were determined using ImageJ software.

MLCL AT-1 enzyme activity assay

Using mitochondria from healthy and BTHS lymphoblasts, MLCL AT-1 enzyme activity was measured as described previously (32, 33).

Phospholipid isolation, sensitive lipid phosphorous analysis, and MS analysis

Phospholipids were isolated from whole cell lysates as described, and using two-dimensional TLC, phospholipids were separated as described (57). To obtain CL mass, CL was isolated from TLC plates and measured using the sensitive lipid phosphorous assay as described (58). For molecular species analysis, cell pellets were resuspended in PBS, lipids were extracted, and CL mass and species composition were determined as described with modifications (59). Lipid extracts were dried down under N₂ and resuspended in Solvent A (acetonitrile/water, 60:40, v/v). Lipid extracts were injected into an Ascentis® Express C₁₈ HPLC column (15 cm × 2.1 mm, 2.7 μm; Supelco Analytical) held at 45 °C by a Shimadzu CTO-20 AC column oven. The mobile phase consisted of Solvent A and Solvent B (isopropanol/acetonitrile, 90:10, v/v) containing 10 mM ammonium formate and 0.1% formic acid. Solvent A was initially at 32% until 4.00 min when it was increased to 45%, at 5.00 min to 52%, at 8.00 min to 58%, at 11.00 min to 66%, at 14.00 min to 70%, at 18.00 min to 75%, and at 21.00 min to 97% before decreasing Solvent B to 32% at 25.10 min until 30.10 min when the run was stopped. The flow rate was held at 260 μl/min for the duration of the run. 10 ng of internal standard were added to samples during extraction. Mass spectral analyses of phospholipids were performed using an AB Sciex (Foster City, CA) 4000 QTrap hybrid quadrupole linear ion trap mass spectrometer equipped with a Turbo V ion source. Negative ionization was used for CL utilizing the following settings: curtain gas, 10 p.s.i.; gas source 1, 26 p.s.i.; gas source 2, 30 p.s.i.; ion spray voltage, −4500 V; collisionally activated dissociation, high; temperature, 500 °C; interface heater, on; declustering potential, −80 V; collision energy, −60 V; entrance potential, −10 V; and collision cell exit potential, −20 V. Data are expressed as the percentage of each CL molecular species peak intensity as a percentage of the peak intensities from all CL molecular species.
Cellular mitochondrial protein was obtained as described above. Once mitochondria were obtained, 100 μg were treated with 50 μl of sample buffer (containing 0.2% n-dodecyl β-D-maltoside, mixed by pipetting, and placed on ice for 15 min. The samples were then centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant, or mitochondrial membrane fraction, was collected and stored at −80 °C. To perform BN PAGE, 1 μl of Coomassie® G-250 additive (charge shift molecule) was added to 20 μl of mitochondrial membrane fraction, and the mixture was then placed into each well of a 3–12% gradient acrylamide-bisacrylamide gel. To perform the electrophoresis, 1 × running buffer (50 mM BisTris and 50 mM Tricine, pH 6.8) was added to the outer chamber (600 ml) of the XCell™ Surelock™ Mini-Cell, and either the dark blue cathode buffer (1 × running buffer with 0.02% Coomassie G-250) or light cathode buffer (1 × running buffer with 0.002% Coomassie G-250) was added to the inner chamber (200 ml). The gel was run at 150 V for 1.5 h and then at 250 V for the last 30 min (electrophoresis performed at 4 °C). If performing in-gel activity assays, the dark cathode buffer is initially used. However, after the dye front has migrated one-third of the way down the gel, the run is paused, and the dark cathode buffer is replaced with 200 ml of prechilled light cathode buffer, and the run is then continued. Coomassie-stained gels (dark cathode buffer used throughout the entire run) had to undergo a fixing and destaining step to be able to visualize proteins. Immediately after the electrophoresis run, the gel was fixed by submerging it in a 40% methanol and 10% acetic acid solution (in a glass container with a lid) and heated using a microwave (medium heat) for 45 s. The gel was then placed in a rocker for 15 min with the lid covering the entire run) had to undergo a fixing and destaining step to be able to visualize proteins. Immediately after the electrophoresis run, the gel was fixed by submerging it in a 40% methanol and 10% acetic acid solution (in a glass container with a lid) and heated using a microwave (medium heat) for 45 s. The gel was then placed in a rocker for 15 min with the lid covering the container. After this time, the fixing solution was replaced by a destaining solution (8% acetic acid), and the gel was placed on a rocker for at least 2 h (an overnight incubation was sometimes required to visualize the proteins on the gel). Images were captured using a Chemidoc MP imaging system using a white light conversion screen or digital photography.

In-gel activity assay (complex I)

To detect CI activity, BN PAGE was performed as described above. After electrophoresis, the gel was submerged in a solution containing 5 mM Tris, pH 7.4, 0.3 mM β-NADH, and 0.1 mM nitro blue tetrazolium and placed in a rocker at room temperature for 2 h. To stop the reaction, the gel was washed twice with double-distilled H2O and then placed in 60 ml of fixing solution (40% methanol and 10% acetic acid) for 15 min. This process also helps remove some of the residual Coomassie stain that remains after running the gel. After 15 min, the gel was then placed in a destaining solution (8% acetic acid) for 1 h.

Mitochondrial OCR measurements

Lymphoblasts are nonadherent cells that must be immobilized to perform mitochondrial function experiments using the Seahorse XF 24 analyzer. Cell-Tak is used to prepare an adherent monolayer of cells in each well of an XF 24 microplate. To coat the XF 24 microplates, Cell-Tak was first diluted in a 0.1 M solution of sodium bicarbonate to a concentration of 22.4 µg/ml. To each of the 24 wells, 50 µl of the diluted Cell-Tak was added (1.12 µg/well) followed by a 20-min incubation period at room temperature. After 20 min, the Cell-Tak was aspirated, and the wells were rinsed once with 200 μl of UltraPure DNase/RNase-free water. The wells were allowed to dry for at least 10 min prior to seeding cells. Lymphoblasts were resuspended in 1 ml of XF assay medium (modified DMEM) containing 1 mM sodium pyruvate and 11 mM D-glucose (medium was warmed to 37 °C, pH 7.4, and filter-sterilized using a 0.22-µm filter before use). Cells were counted using a hemocytometer and then diluted to achieve a concentration of 3000 cells/µl for healthy lymphoblasts and 2000 cells/µl for BTHS lymphoblasts. This difference is due to the difference in cell size that exists between the smaller healthy cells and the BHTS cells (which are about 35–40% larger). Cell number optimization experiments demonstrated that these respective concentrations are necessary to achieve a monolayer of cells in each well of the XF analyzer plate. A volume of 100 µl of the diluted samples was added to corresponding wells of a Cell-Tak–coated XF analyzer 24-well plate. The plate was centrifuged at room temperature for 10 min at 1200 rpm and then placed at 37 °C in a CO2-free incubator for a maximum of 20 min. During this incubation period, an XF sensor cartridge, calibrated overnight at 37 °C in a CO2-free incubator, was loaded with oligomycin (port A, 75 µl of a 10 µM solution), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (port B, 83 µl of a 10 µM solution), and rotenone/antimycin A (port C, 93 µl of 10 µM solution). The goal is to achieve a final concentration of 1 µM for each of the drugs in each plate well. Once loaded, the sensor cartridge was placed in the XF analyzer and calibrated. After the incubation period, the plate wells were topped off with 575 µl of the supplemented XF assay medium, and the plate was then placed in the XF 24 analyzer.

Glycolysis measurement

Healthy and BTHS lymphoblasts were resuspended in 1 ml of XF assay medium (unbuffered DMEM, 0 mM glucose, warmed to 37 °C, pH 7.35, filter-sterilized using 0.22-m filter) supplemented with 1-glutamine (final concentration, 2 mM). The cells were seeded onto the Cell-Tak–coated XF microplate as described above and incubated at 37 °C in a CO2-free incubator for 1 h. During this time, an XF sensor cartridge, calibrated overnight at 37 °C in a CO2-free incubator, was loaded with oligomycin (port A, 75 µl of a 10 µM solution), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (port B, 83 µl of a 10 µM solution), and rotenone/antimycin A (port C, 93 µl of 10 µM solution). The goal is to achieve a final concentration of 1 µM for each of the drugs in each plate well. Once loaded, the sensor cartridge was placed in the XF analyzer and calibrated. After the incubation period, the plate wells were topped off with 575 µl of the supplemented XF assay medium, and the plate was then placed in the XF 24 analyzer.

ROS detection via flow cytometry

For the detection of ROS, cells were incubated with a 5 µM concentration of the mitochondrion-specific superoxide indicator MitoSOX as described (60). After a 20-min incubation at 37 °C (protected from light), cells were washed twice with and resuspended in Hank’s balanced salt solution containing calcium and magnesium, and then fluorescence was detected using a BD LSRII flow cytometer and FACSDiva software (488-nm excitation in the FL2 and FL3 channels). As a positive
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control, additional samples were incubated with MitoSOX plus 10 μM antimycin A. Antimycin A is a known inducer of superoxide production.

Statistical analysis

Data are expressed as mean ± S.D. with the number of experiments indicated. Comparisons between groups were performed by unpaired, two-tailed Student t test or one-way analysis of variance using Student–Newman–Keuls post hoc analysis where appropriate. The level of significance was defined as p < 0.05.

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