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LETTER TO THE EDITOR

Lysosomal inhibition attenuates peroxisomal gene transcription via suppression of PPARA and PPARGC1A levels

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ABSTRACT

Lysosomes influence dynamic cellular processes such as nutrient sensing and transcriptional regulation. To explore novel transcriptional pathways regulated by lysosomes, we performed microarray analysis followed by qPCR validation in a mouse hepatocyte cell line, AML12, treated with bafilomycin A_1 (lysosomal v-type H^+-translocating ATPase inhibitor). Pathway enrichment analysis revealed significant downregulation of gene sets related to peroxisomal biogenesis and peroxisomal lipid oxidation upon lysosomal inhibition. Mechanistically, pharmacological inhibition of lysosomes as well as genetic knockdown of *Tfeb* led to downregulation of the peroxisomal master regulator PPARA and its coactivator PPARGC1A/PGC1α. Consistently, ectopic induction of PPARA transcriptional activity rescues the effects of lysosomal inhibition on peroxisomal gene expression. Collectively, our results uncover a novel metabolic regulation of peroxisomes by lysosomes via PPARA-PPARGC1A transcriptional signalling.

Abbreviations: *Acox1*: acyl-Coenzyme A oxidase 1, palmitoyl; *Acot*: acyl-CoA thioesterase; ACAA: acetyl-Coenzyme A acyltransferase; ABCD3/PMP70: ATP-binding cassette, sub-family D (ALD), member 3; BafA1: bafilomycin A_1; *Crot*: carnitine O-octanoyltransferase; CTSB: cathepsin B; *Decr2*: 2-4-dienoyl-Coenzyme A reductase 2, peroxisomal; *Ech1*: enoyl coenzyme A hydratase 1, peroxisomal; *Ehhadh*: enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase; FDR: false discovery rate; Hsd17b4: hydroxysteroid (17-beta) dehydrogenase 4; NES: normalized enrichment score; NOM: nominal; Pex: peroxin; PPARA: peroxisome proliferator activated receptor alpha; PPARGC1A: peroxisome proliferator activated receptor, gamma, coactivator 1 alpha; TFEB: transcription factor EB.

Introduction

Lysosomes are the terminal cellular degradation sites where macromolecules such as carbohydrates, lipids, and proteins are catabolized to meet cellular energy needs [1,2]. These catabolic events occur in the highly acidic lumen (pH of ~4.5–5.0) of the lysosome, and are separated from the cytoplasm by a single lipid bilayer. Maintenance of the acidic environment required for lysosomal internal hydrolytic activities\ requires the activity of a vacuolar H+-ATPase (v-ATPase) that constantly pumps in protons (H^+ ions) across its limiting membrane [3]. It appears that lysosomes are not only limited to degradative functions but also may communicate closely with other cellular organelles to maintain metabolic homeostasis. Recent studies have identified a retrograde signalling pathway that extends from lysosomes to the nuclear transcriptional machinery [4]. This lysosome-to-nucleus signalling may be critical for the execution of mitochondrial lipid catabolic programs in the liver [5]. Currently, little is known about other lysosomal signalling mechanisms that enable lysosomes to communicate with various cellular compartments.

In this study, we utilized a genomic approach to uncover the effect of lysosomes on the cellular transcriptome when lysosomal activity was pharmacologically blocked. Surprisingly, pathway analysis of our dataset identified several peroxisomal gene sets that were down-regulated due to lysosomal inhibition. These findings suggested that lysosomes might regulate lipid metabolism and cellular redox maintenance through their actions on peroxisome activity [6]. In particular, we found that inhibition of cellular lysosomal function decreased the expression of genes involved in peroxisomal biogenesis and lipid catabolism through effects on PPARGC1A-PPARA mediated transcription.

Results

To explore the effect of lysosomal inhibition on cellular transcriptome and signalling pathways, mouse hepatic cells AML12 cells were treated with bafilomycin A_1 (BafA1). BafA1 increases lysosomal pH by binding to the interface between the transmembrane helices of ATP6V0C/subunit c of the V_0 domain of the v-ATPase, preventing helical swivelling and inhibiting the latter's activity [7]. We initially observed BafA1 inhibition of lysosomal activity via the loss of LysoTracker staining (Figure 1(a)) and

reduced activation of CTSB (cathepsin B), a lysosomal protease activated in acidic pH (Figure 1(b,c)). We then performed micro-array analyses on AML12 cells treated with or without BafA1. Bioinformatics pathway enrichment analysis revealed several gene datasets involved in peroxisome biogenesis, peroxisomal lipid metabolism, and PPARA signalling pathways as significantly down-regulated in cells treated with BafA1 (Figure 1(d)) and Tables S1–S3).

We next validated the genes enriched in the KEGG and Reactome pathways by performing qRT-PCR. Our results showed significant down-regulation of several peroxisomal genes involved in peroxisomal biogenesis (Pex genes) and lipid metabolism, including Ppara itself, in BafA1-treated cells (Figure 1(e)). In concordance, peroxisomal markers such as ABCD3/PMP70 and CAT (catalase) expression and enzyme activity were significantly decreased in BafA1-treated cells (Figures 1(f–h) and S1). These findings strongly suggested a link between lysosomal activity and peroxisomal gene transcription.

Because we observed decreased levels of peroxisomal gene expression after lysosomal inhibition, we examined the protein levels of the master peroxisomal transcription factor, PPARA, and its co-activator, PPARGC1A [8]. We found that PPARA protein expression was lower in cells treated with BafA1, consistent with its decrease in mRNA level (Figure 1(i,j)). We also observed that the expression of several major target genes of PPARA [9,10], such as Acox1 and Cpt1a were decreased at both RNA and protein levels (Figures S2 & 1(i,j)), suggesting that PPARA-transcriptional activity was reduced after lysosomal inhibition. PPARGC1A is a major coactivator of PPARA and is required for peroxisomal biogenesis in both a PPARA-dependent and PPARA-independent manner [11]. Similar to our findings on PPARA, we found a significant time-dependent decrease in PPARGC1A protein expression in BafA1-treated cells (Figure 1(k,l)). Moreover, other lysosomal inhibitors such as concanamycin A (ConA) and chloroquine (CQ) also showed similar inhibitory effects on PPARGC1A levels (Figure S3). Additionally, the effects of BafA1 on peroxisomal gene expression as well as on PPARA and PPARGC1A levels were also mimicked by the knockdown of the master lysosomal biogenesis regulator Transcription factor EB (Tfeb) (Figure S4).

We next examined whether downregulation of PPARGC1A protein by BafA1 was primarily due to its decreased transcription as was observed in the case of Ppara. To our surprise, Ppargc1a mRNA levels increased after 24 h of BafA1 treatment but later decreased after 72 h of treatment (Figure S5(a)). We thus examined whether the downregulation of PPARGC1A protein at early time periods (24 h post BafA1 treatment) was due to increased proteasomal degradation. Indeed, the attenuation in PPARGC1A protein levels in BafA1-treated cells was prevented by the proteasome inhibitor, MG132 (Figure S5(b)). These results suggest that during lysosomal inhibition, the ubiquitin-proteasomal pathway is involved in the early downregulation of PPARGC1A protein; however, the decrease in Ppargc1a mRNA expression may also contribute to the downregulation of PPARGC1A protein at later time periods.

Furthermore, in order to provide direct evidence for the involvement of PPARGC1A-PPARA transcriptional signalling pathway in BafA1 suppression of peroxisomal gene expression we overexpressed PPARA and PPARGC1A (Figure S6). Our results clearly showed that PPARA ligand rescued the suppressive effect of BafA1 on peroxisomal gene transcription in PPARA overexpressing cells, and induced a further increase in their transcription when PPARGC1A was co-expressed in these latter cells (Figure 1(m)).

Discussion

In this study, we uncovered a signalling pathway that links lysosomes to peroxisomes, 2 intracellular organelles that were discovered by de Duve more than 50 y ago [12]. The conventional model considers lysosomes as intracellular organelles that primarily recycle, degrade, and release building blocks such as amino acids and free fatty acids after autophagosome-lysosome fusion. However, lysosomes may be more complex than previously thought, and able to modulate the function of other organelles. In this connection, previous studies showed that lysosomes regulated nuclear transcriptional signalling to increase their own biogenesis as well as lipid catabolism within the cell [4,5].

Here, we found that cellular pathways containing genes involved in peroxisomal biogenesis and peroxisomal lipid metabolism were significantly down-regulated after lysosomal inhibition, including the peroxin (Pex) genes that are involved in peroxisomal biogenesis and growth [13]. Among the peroxins that were decreased by BafA1 treatment, PEX7 and PEX26 are required for the peroxisomal matrix protein import, PEX11A is mainly involved in peroxisome division, and PEX19, which has different functions in yeast and mammals (budding of pre-peroxisomal vesicles and insertion of the peroxisomal membrane proteins in the peroxisomal membrane, respectively [14–17]. We further confirmed that lysosomal inhibition reduced peroxisomal biogenesis by observing decreases in the ABCD3/PMP70 expression [18] and CAT activity [19]. Similarly, gene expression of several peroxisomal lipid-metabolizing enzymes such as Acox1/2, Crot, Acaa1, Hsd17b4, etc. was reduced. Peroxisomal β-oxidation in rodents, especially in the liver, is highly inducible in a PPARA (peroxisome proliferator activated receptor alpha)-dependent manner in animals fed with peroxisome proliferators [20]. Of note, several of the downregulated peroxisomal genes that we observed were PPARA transcriptional targets. Consistent with this observation, we found that PPARA expression also was transcriptionally suppressed in BafA1-treated cells, and likely contributed to the decrease in peroxisomal gene expression and activity. The precise mechanism for how lysosome inhibition represses PPARA expression is not known; however, it is possible that lysosomal degradation of lipids could provide endogenous ligands for PPARA and thus provide an additional layer for regulation of PPARA activity.

PPARGC1A is the major coactivator in PPAR-mediated transcriptional induction of genes involved in lipid oxidation [21]. Expression of PPARGC1A is itself highly regulated at both the transcriptional as well as the post-translational levels [22]. Our results showed that lysosomal inhibition down-regulated PPARGC1A protein expression despite up-regulating its mRNA level during the early time periods. This decrease in PPARGC1A involved proteasomal degradation since proteasomal inhibition completely abolished the effect of lysosomal inhibition. Because lysosomal inhibition may lead to a general increase in proteasomal activity [23], it is
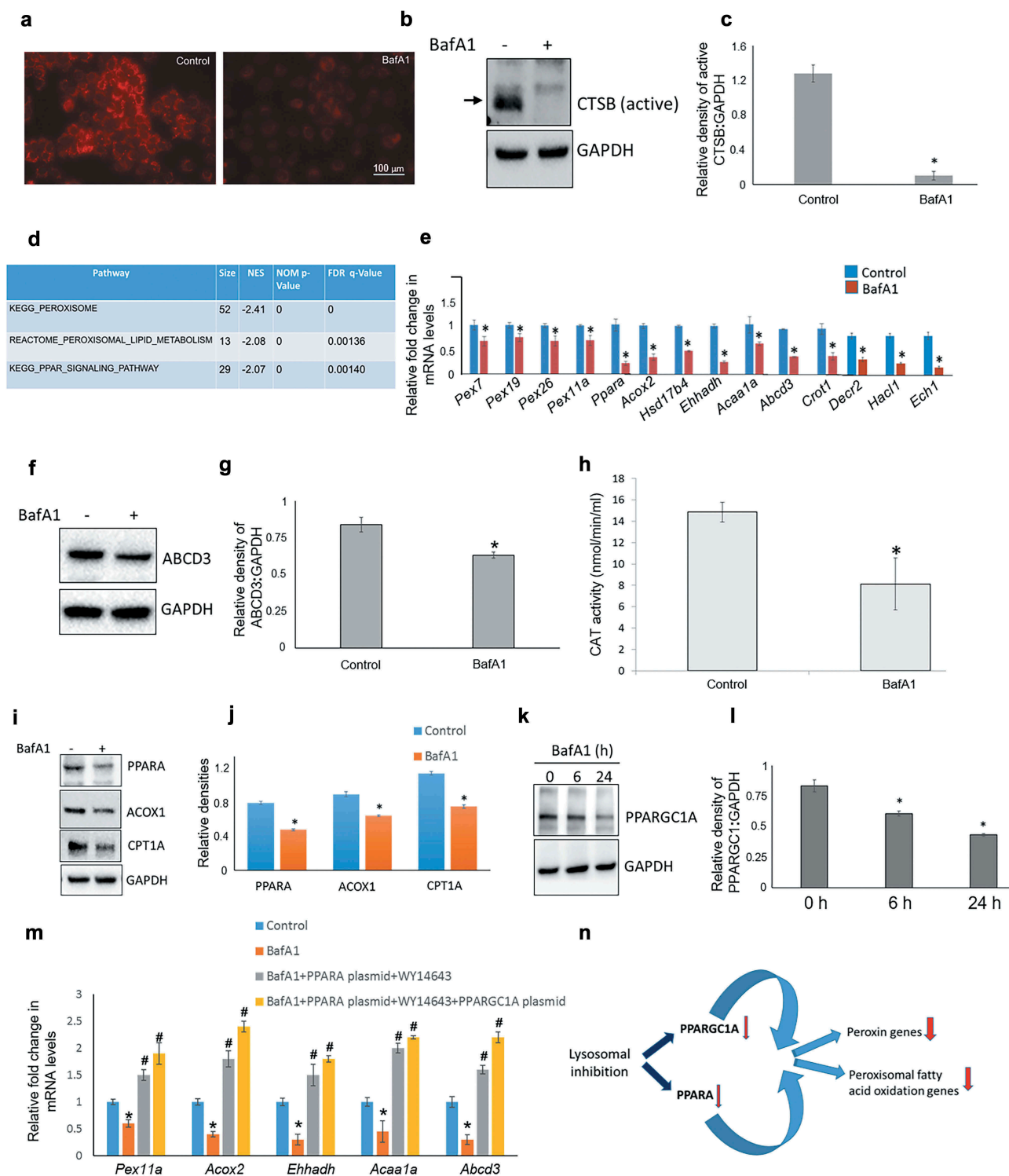
Figure 1. Lysosomal inhibition impairs peroxisomal gene expression. (a) Lysotracker staining of AML12 cells treated with ± BafA1(10 nM/24 h). (b and c) Representative immunoblot and densitometry showing cellular levels of active CTSB± BafA1 (10 nM/24 h). Values are means ±SD (n = 3), *P < 0.05. (d) Gene ontology-KEGG & Reactome Pathway analysis of the genes obtained from AML12 cells ± BafA1 (10 nM/24 h). (e) qRT-PCR validation of peroxisomal genes ± BafA1 (10 nM/24 h) in AML12 cells. Values are means ±SD (n = 3), *P < 0.05. (f and g) Representative immunoblot and densitometry analysis showing cellular levels of active ABCD3 levels ± BafA1 (10 nM/24 h). Values are means ±SD (n = 3), *P < 0.05. (h) Catalase (CAT) activity ± BafA1 (10 nM/24 h) in AML12 cells. Values are means ±SD (n = 5), *P < 0.05. (i and j) Representative immunoblot and densitometry showing cellular levels of PPARA, ACOX1 and CPT1A ±BafA1 (10 nM/24 h). Values are means ±SD (n = 3),*P < 0.05. (k and l) Representative Immunoblot and densitometry showing protein levels of PPARGC1A ±BafA1 (10 nM) for indicated time of treatment. Values are means ±SD (n = 3), *P < 0.05. (m) qRT-PCR results showing the effect of PPARA, PPARGC1A overexpression on peroxisomal gene transcription in the presence of BafA1. Briefly, cells were transfected with 500 ng of PPARA- and/or PPARGC1A-expressing plasmids using Lipofectamine 3000 and after 24 h of transfection BafA1 (10 nM) was added with or without WY14643 (15 μM, PPARA agonist) for the next 24 h before RNA isolation. Values are means ±SD (n = 3), *P < 0.05 between BafA1 vs Control, #P < 0.05 between PPARA/PPARGC1A overexpression vs. BafA1 group. (n) Model for lysosomal inhibition-induced peroxisomal defect. Based on our results we propose that pharmacological inhibition of lysosomes leads to reduced PPARGC1A and PPARA levels in cells. This reduction may explain the observed attenuation in the expression of peroxisomal genes.

not clear whether there is a selective degradation of PPARGC1A in BafA1-treated cells. Interestingly, in contrast to BafA1, *Tfeb* knockdown significantly decreased *Ppargc1a* mRNA levels, most likely due to TFEB direct regulation of *Ppargc1a* expression as shown previously [5,24].

Lysosomes play a key role in regulating peroxisomal number in cells via pexophagy [24]. Moreover, there is observed impairment of peroxisomal function in the lysosomal storage disorders, Neimann-Pick disease and X-linked adrenal leukodystrophy [25]. It is possible that inhibition of peroxisomal biogenesis due to lysosomal inhibition may be an adaptive response to avoid accumulation of peroxisomes under stress, especially when there may be an underlying defect in lysosome-mediated pexophagy as has been shown previously for mitophagy [26–28]. In mammals, very long chain fatty acids and their derivatives are β-oxidized specifically by peroxisomes [6]. In animal models of genes involved in peroxisomal β-oxidation, peroxisomes appear to play a critical role in energy metabolism, since their deficiency can cause hepatic steatosis, steatohepatitis, and liver cancer [20]. Additionally, it is possible that hepatic lipid accumulation caused by pharmacological inhibition of lysosomal activity [29] may be partly due to defective peroxisomal lipid handling.

The novel connection between lysosomal function and peroxisomal gene transcription via PPARGC1A-PPARA nuclear receptor activity (Figure 1(n)) raises the possibility that peroxisomal activity can be enhanced by increasing lysosomal activity. This strategy could potentially be applied to disorders where there is impairment of peroxisomal function such as Neimann-Pick disease and X-linked adrenal leukodystrophy. Finally, PPARGC1A also regulates several other nuclear hormone receptors, so we speculate that lysosomal inhibition could modulate other cellular and metabolic pathways mediated by these other nuclear receptors [30].

Material and methods

Reagents

Bafilomycin A$_1$ (B1793), concanamycin (27689), chloroquine (C6628), MG132 (M8699), WY14643 (C7081) and anti-PPARGC1A antibody (SAB2500781) were purchased from SIGMA-ALDRICH. Anti-PPARA antibody (PA1-822A), LysoTracker Red DND-99 (L7528) and DMEM-F12 (11330057) were from ThermoFisher Scientific. Anti-CPT1A (ab128568), anti-GAPDH (ab9485) and anti-ACOX1 (ab9964) antibodies were from ABCAM. Ppara (NM_011144) plasmid was from ORIGENE, GFP-PGC1 was a gift from Bruce Spiegelman (Addgene, 4).

Cell culture

AML12 (CRL-2254) cells were maintained at 37°C in DMEM-F12 1:1 containing 10% fetal bovine serum, 1x ITS (ThermoFisher Scientific, 41400), 10 nM dexamethasone and 1x penicillin/streptomycin in a 5% CO_2 humidified incubator. For siRNA transfection, cells were transfected using RNAiMAX (ThermoFisher Scientific, 13778150) with *Tfeb* (ThermoFisher Scientific, 4390771) siRNA (10 nM) for 96 h followed by harvesting cells

for RNA and protein extraction. Lipofectamine 3000 (ThermoFisher Scientific, L3000001) was used for plasmid transfections.

Microarray and pathway analysis

Gene expression microarray profiling was performed using GeneChip Mouse Gene 2.0 ST Array (Affymatrix) on pool of 3 samples. cRNA generation, labeling and hybridization were performed at Duke-NUS Genome Biology Facility, Duke-NUS Graduate Medical School, Singapore. Gene expression signals were quantile normalized, and differentially expressed genes were identified via analysis of variance, using treatment specific contrasts (Partek Genomics Suite software, version 6.6). Statistical significance of differentially expressed genes was ascertained in terms of the false discovery rate. Pathway enrichment analysis was conducted via the Gene Set Enrichment Analysis tool using a list of KEGG and Reactome pathways extracted from the Molecular Signatures Database. Significance of pathway enrichment was ascertained by permutation testing of gene sets and calculation of the false discovery rate (FDR q value <0.050).

RNA isolation and real-time PCR

Total RNA was isolated and qRT-PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, 204141) in accordance with the manufacturer's instructions. Primer sequence available upon request.

Western blotting

Cells or tissue samples were lysed using CelLytic™ M Cell Lysis Reagent (Sigma, C2978) and immunoblotting was performed as per manufacturer's guidelines (Bio-Rad Laboratories, USA). Image acquisition was done using ChemiDoc (Bio-Rad ChemiDoc™ MP System, 1708280). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

LysoTracker staining

Cells were grown on glass coverslips and treated with BafA1 for the required period. Thereafter, cells were incubated with 100 nM of LysoTracker Red DND-99 for 15–30 min at 37°C, followed by three phosphate-buffered saline (PBS) washes, and then immediately observed under a fluorescence microscope.

CAT assay

CAT activity was measured using commercial kit (Catalase Assay Kit; Cayman Chemicals, 707002).

Calculations and statistics

Results are expressed as mean ± SD. The statistical significance of differences (*$P < 0.05$) was assessed by unpaired student t-test when comparing different groups.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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