Cyclic guanosine monophosphate inhibits the palmitoylation of LETM1 and downregulates the activity of AKT in human pulmonary artery smooth muscle cells

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Abstract

Cyclic guanosine monophosphate (cGMP) is a drug target for the treatment of pulmonary arterial hypertension (PAH). However, the molecular mechanism of cGMP in the pathogenesis of PAH remains unclear, and long-term use of cGMP target drugs leads to drug resistance. We tried to explore the new targets downstream of cGMP signaling and develop novel therapies for PAH. We combined mass spectrometry (MS)-based quantitative proteomics with click-chemistry-probes to identify palmitoylated candidate proteins. S-palmitoylation assay was utilized to test whether the palmitoylation level of the candidates was regulated by cGMP. We identified palmitoylated proteins in human pulmonary artery smooth muscle cells (HPASMCs). Functional enrichment of the palmitoylated proteins showed that 52 proteins were involved in transmembrane transport, of which 7 were engaged in mitochondrial transport. S-palmitoylation assay in vitro demonstrated that the inner mitochondrial membrane protein, LETM1, was palmitoylated in the cytoplasmic domain at C-terminus. More importantly, cGMP could inhibit the palmitoylation level of LETM1, which in turn suppressed the activity of AKT regulated by LETM1. Our study found that the palmitoylation of LETM1 was the new target downstream of cGMP signaling. The palmitoylated site of LETM1 may provide a new direction for the development of novel therapies for PAH. Moreover, the palmitoylation level of LETM1 may be a new biomarker for the analysis of prognosis.

Introduction

Cyclic guanosine monophosphate (cGMP) is the second messenger of nucleotides, which was first identified about a half a century ago [1]. cGMP originates from guanylyl cyclases (GC) catalyzed by natriuretic peptide or nitric oxide (NO) and transduces its effects by downstream phosphodiesterases (PDE) and protein kinases [2]. cGMP regulates many cellular functions, ranging from contractility to growth. The NO/cGMP signaling pathway is essential for the maintenance of vascular tone and dysfunction of the cGMP signaling leads to a number of cardiovascular diseases, such as pulmonary arterial hypertension (PAH) [3]. PAH is a progressive disease characterized by elevated pulmonary arterial pressure, resulting in pulmonary vasoconstriction, thrombosis in situ, and even right heart failure and death [4]. cGMP is the main target of drug therapy for PAH, but long-term use of cGMP targeted drugs is prone to drug resistance. At the same time, these drugs promote peripheral vasodilation, which will lead to the side effects of systemic hypotension. In addition, the molecular mechanism of cGMP in the pathogenesis of PAH remains elusive.

Protein palmitoylation is a post-translational modification of proteins with fatty acids, which plays a significant part in eukaryotic physiology and human pathology by regulating protein localization, trafficking, stability, assembly, and protein-protein interaction [5, 6]. Protein palmitoylation involves the covalent connection of a 16-carbon fatty acid moiety, palmitate, and amino acid residues, such as cysteines (S-palmitoylation), serines/threonines (O-palmitoylation) and lysines (N-palmitoylation) [7]. Among them, only S-palmitoylation is reversible, which is catalyzed by protein acyl-transferases with Asp-His-His-Cys (DHHC) motifs and removed by acyl-protein thioesterases [8]. Based on the strict regulation for activity, researchers pay more attention to S-palmitoylation in the last 30 years and detect more than

400 palmitoylated proteins, including both soluble proteins and integral membrane proteins [9]. For soluble proteins, such as H-Ras and N-Ras, palmitoylation facilitates the localization to lipid rafts and plasma membranes by enhancing the hydrophobicity of them [10]. For integral membrane proteins, palmitoylation occurs not only in cytoplasmic tails or loops but also in the vicinity or within transmembrane domains [11]. Due to the difficulties in analyzing a lipid modification and dealing with transmembrane domains, more studies are now focused on the cytoplasmic, including receptors, channels and transporters. Numerous channels or transporters are essential for controlling the physiology and pathology of tissues through the transmembrane transport of ions, proteins, metabolic products, etc., and the activities of some of them are regulated by palmitoylation. For example, P2X₇ is a non-selective cation channel activated by extracellular ATP. Cryo-electron microscopy structures of rat P2X₇ show that palmitoylation is observed in the cytoplasmic element, c-cysteine anchor, which prevents desensitization by anchoring the pore-lining helix to the membrane [12]. Another case is about the transmembrane sodium-calcium exchanger 1 (NCX1), which facilitates the bidirectional transport of calcium levels by the gradient of transmembrane sodium. The palmitoylation of NCX1 is localized in the intracellular loop and the inactivation of NCX1 is controlled by modifying the NCX1 dimer's structure [13]. Based on the regulatory mechanism of palmitoylation on transmembrane transport, the pharmacological targets of palmitoylation sites can be used as a way of clinical treatment of related diseases. Considering the key role of palmitoylation and the limitations of cGMP targeted therapy for PAH, the present study aimed to identify the cGMP-regulated palmitoylated proteins.

In this study we combined mass spectrometry (MS)-based quantitative proteomics with click-chemistry-probes to quickly identified palmitoylated candidate proteins that maybe regulated by cGMP in cell lines [14]. The functional palmitoylated sites may provide a new direction for the development of novel therapeutic methods for PAH. Moreover, the palmitoylation levels of specific proteins regulated by cGMP may represent new biomarkers for analyzing prognosis.

Materials And Methods

Cell culture.

HPASMCs (ScienCell Research Laboratories, California, USA) were isolated from human pulmonary arteries [15] and cultured in the smooth muscle cell (SMC) medium (ScienCell Research Laboratories, Carlsbad, USA) supplemented with 2% fetal bovine serum and 1% SMC growth supplement. This study was approved by the Beijing Hospital Ethics Committee (BHEC) with the registration No. of 2017BJYYEC-108-05. Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 4.5 mg/ml glucose. All the cells were incubated at 37°C in a humidified normoxia condition (21% O₂, 5% CO₂ and 74% N₂).

Construction of plasmids and DNA transfection.

To construct the expression plasmids for LETM1-Flag, a DNA fragment containing LETM1 was inserted into pCDNA3.1-Flag-c. LETM1^{C552A} mutant was constructed by PCR using the primers (Letm1C552A-F: 5'-CCTCAGCGATGCCGCCTCTAAGCTG-3' and Letm1C552A-R: 5'-GCGGCATCGCTGAGGATGTCGATTTC-3') and cloned into pCDNA3.1-Flag-c. LETM1-Flag and LETM1^{C552A}-Flag were transiently expressed in Hela cells and DNA transfection was performed using lipo3000 (Invitrogen) according to the manufacturer's instructions.

Antibodies and reagents.

The antibodies used in the present study were as follows:

rabbit anti-LETM1 (dilution 1:2,000; cat. no. 16024-1-AP), rabbit anti-THEM4 (dilution 1:2,000; cat. no. 14692-1-AP), mouse anti-GAPDH (dilution 1:2,000; cat. no. 60004-1-Ig), rabbit anti-Flag (dilution 1:2,000; cat. no. 20543-1-AP), mouse anti-AKT (dilution 1:2,000; cat. no. 60203-1-Ig), mouse anti-pS473-AKT (dilution 1:2,000; cat. no. 66444-1-Ig). All of the antibodies were purchased from Protein Tech Group, Inc.

The reagents used in the present study were as follows:

protease inhibitor cocktail (Roche Group), protein (A/G) ultraLink resin (Thermo Fisher Scientific, Inc.), azide agarose beads (Nanocs), thiopropyl sepharose 6B (Sigma-Aldrich; Merck KGaA), hydroxylamine (Sigma-Aldrich; Merck KGaA), N-ethylmaleimide (NEM; Sigma-Aldrich; Merck KGaA), Tris (2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich; Merck KGaA), Tris [(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA; Sigma-Aldrich; Merck KGaA), 8-Br-cGMP (Sigma-Aldrich; Merck KGaA).

Immunoprecipitation (IP) and western blot analysis.

HPASMCs were harvested and lysed (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail) on ice for 30 min. IP and western blots were performed as previously described [16].

Protein S-palmitoylation assay in cells.

The HPASMCs were treated with 8-Br-cGMP (1mM) or H₂O (control). Hela cells were transfected with pCDNA3.1-LETM1-Flag and pCDNA3.1-LETM1^{C552A}-Flag. After 24 h, both the HPASMCs and Hela cells were harvested for S-palmitoylation assay. The S-palmitoylation assay was performed as previously described [17] with minor modifications. In brief, the cells were washed in PBS and lysed in lysis buffer [10 mM sodium phosphate, 2 mM Na₂-EDTA, 0.32 M sucrose, 1% Triton X-100, protease inhibitor cocktail; pH 7.4] on ice for 30 min. Then, the lysates were added with N-ethylmaleimide (50 mM) and immunoprecipitated at 4°C overnight utilizing protein A/G resin preloaded with LETM1 or Flag antibody. Next day, the protein A/G resin was washed three times in lysis buffer and incubated with elution buffer (1% SDS, 10 mM sodium phosphate, 2 mM Na₂-EDTA, 0.32 M sucrose) at 50°C for 5 min to release

LETM1, LETM1-Flag or LETM1^{C552A}-Flag. Eluted samples were divided into two equal portions. One was treated with 1 M hydroxylamine (pH 7.4) and thiopropyl sepharose 6B and the other was treated with 1M Tris·HCl (pH 7.4) and thiopropyl sepharose 6B as the negative control. After a 2-h incubation at room temperature, sepharose beads were washed three times with washing buffer (10 mM sodium phosphate, 2 mM Na₂-EDTA, 0.32 M sucrose, 1% Triton X-100, 500 mM NaCl and 0.2% SDS). Western blots was performed to determine the presence of LETM1, LETM1-Flag or LETM1^{C552A}-Flag.

Synthesis of palmitate probe, metabolic labeling and click chemistry reaction.

The ω -alkynyl-palmitate acid analogue, Alk-C16, was synthesized as previously described [18] and dissolved in DMSO to generate a 50-mM stock solution and stored at 80°C. Before cell treatment, AlkC16 was dissolved in SMC medium supplemented with 5% BSA (fattyacid free) and 1% SMC growth supplement at a final concentration of 100 μ M, and then sonicated for 15 min at room temperature. In addition, an equal volume of DMSO was added to the same medium as a negative control. Then, the seeded HPASMCs were incubated in the above medium containing Alk-C16 or DMSO respectively for 24 h at 37°C with 5% CO₂. Then, the cells were harvested and click chemistry reaction were performed as previously described [18]. In brief, the cells were lysed in 500 μ l lysis buffer (1% Nonidet P-40, 150 mM NaCl, 100 mM sodium phosphate and protease inhibitor cocktail; pH 7.5) for 30 min at 4°C. Then, the protein extracts were subjected to the click-chemistry reaction for 1 h at room temperature by the addition of the following reagents in order: 1 mM azide agarose beads, 1 mM TCEP dissolved in water, 0.2 mM TBTA dissolved in DMSO/tert-butanol (20/80% v/v) and 1 mM CuSO₄ in PBS. Following the reaction, the Alk-C16-conjugated proteins were bound to the azide agarose beads. The beads were washed three times with lysis buffer at room temperature. Then, the proteins bound to the beads were digested for MS analysis.

Proteome analysis.

NanoLCMS/MS analysis, database searching, data processing and statistical analysis were performed as previously described[17, 19]. The steps were as follows.

An Easy nLC1000 (Thermo Fisher Scientific, Inc.) system combined with the LTQ-Orbitrap-Elite (Thermo Fisher Scientific, Inc.) mass spectrometer were applied for proteomic profiling.

The recorded MS spectra was analyzed by utilizing MaxQuant Software (version 1.5.5.1; <https://www.Maxquant.org/>). The MS/MS peak list analysis was performed by searching against a forward and reverse version of the UniProtKB/Swiss-Prot human database (generated from version 2017_05; human taxonomy; 20,316 entries; <http://www.uniprot.org/>). The cutoff of the false discovery rate for peptide and protein identification was set to 0.01, and only peptides with ≥ 7 amino acidic residues were analyzed. Label-free quantitation (LFQ) was performed using the MaxQuant software on the identified razor and unique peptides in order to quantify the identified proteins.

Protein abundances normalized by the LFQ algorithm integrated in MaxQuant were log2-transformed for further analysis. The filtering steps were performed using Microsoft Excel 2010. DanteR (version 1.0.1.1) and Perseus (version 1.5.5.3) were used to perform various types of statistical analysis including log2 transformation, correlation plot, statistical tests and *p*-value adjustments.

Functional analysis.

The functional enrichment analysis of the identified palmitoylated proteins were performed by Metascape web-based platform [20]. Enriched terms with a *p*-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were collected and grouped into clusters based on their membership similarities. "Log10 (*P*)" is the *p*-value in log base 10. Protein-protein interaction (PPI) network construction was performed by STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string-db.org/>) [21].

Results

Identification transport proteins of palmitoylome using AlkC16 in HPASMCs.

In order to identify the palmitoylated protein profiles of HPASMCs, the cells were metabolic labelled by Alk-C16, an alkynyl-palmitic acid. The proteins modified by the alkynyl-palmitic acid were chemoselectively ligated to azide agarose beads by a click chemistry reaction of Cu¹-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC). The application of CuAAC could enrich the palmitoylated proteins on the azide agarose beads. Then, the proteins on the beads were digested by trypsin and subjected to LC-MS/MS and palmitoylome analysis. (Fig. 1)

The results of palmitoylome analysis indicated that a total of 1997 proteins were identified, which exhibited a Mascot score > 2 (*P*<0.05) and peptide > 2. The proteins were subjected to LFQ using MaxQuant software and the LFQ values were positively correlated with the palmitoylation levels. Among these 1997 proteins, 1803 proteins were identified to be palmitoylated preliminarily, and their LFQ values were > 0 in at least 2 replicates. Functional enrichment analysis of 1803 palmitoylated proteins by Metascape showed that 267 of them engaged in transport including vesicle-mediated transport, nucleocytoplasmic transport and transmembrane transport (Fig. 2). Vesicle-mediated transport (R-HSA-5653656) included endocytosis, vacuolar transport, phagocytosis, endosomal transport, cytoskeleton-dependent transport (GO: 0030705, GO: 0008088, GO: 0010970, GO: 0072384, GO: 0099111, GO: 0047496, GO: 0008089, GO: 0048489) and Golgi-ER related transport (GO: 0048193, GO: 0006888, GO: 0006890, R-HSA-199977, R-HSA-8856688, R-HSA-6807878). Nucleocytoplasmic transport (GO: 0006913) engaged in the export from nucleus of RNA, proteins or nitrogen compound. Small molecules transport (R-HSA-382551) involved transmembrane transport of ions, proteins or metabolic products, which we will focus on in the following sections.

Protein-protein interaction network analysis of the small molecules transport proteins.

There were 52 proteins involved in the small molecules transports (R-HSA-382551) (TableS1). The PPI network of them consisted of 52 nodes and 113 interactions (Fig. 3). PPI combined with GO enrichment analysis showed that the function of the transport proteins concentrated on four portions: iron ion transport (GO: 0006826) (purple marks), mitochondrial transport (GO: 0006839) (green marks), anion transport (GO: 0006820) (red marks), low-density lipoprotein particle clearance (GO: 0034383) (yellow marks) (Fig. 3 and TableS2). Previous studies have shown that mitochondrial function inhibition is the key to the development of PAH [22]. Furthermore, it has never been studied how palmitoylation regulates the activity of mitochondrial transport proteins. The proteins involved in mitochondrial transport (GO: 0006839) included AFG3L2, LETM1 (Leucine zipper-EF-hand-containing transmembrane protein 1), PMPCA, MICU2 (Mitochondrial calcium uniporter protein 2), STOML2 (Stomatin-like protein 2), SLC25A5, and SLC25A6. Both AFG3L2 and PMPCA contribute to the mitochondrial proteins import through the cleavage of their peptides [23, 24]. LETM1 is an antiporter that mediated proton-dependent calcium efflux from mitochondrion [25]. MICU2 regulates calcium uptake [26]. SLC25A5 and SLC25A6 mediate the output of ATP and provide fuel for cells [27]. Next, we further verified the palmitoylation of the mitochondrial transport proteins by S-palmitoylation assay in vitro and screened the candidate proteins, whose palmitoylation level was regulated by cGMP in HPASMCs.

LETM1 is a novel palmitoylated protein at C-terminus.

To confirm the palmitoylated proteins involved in mitochondrial transport, we performed S-palmitoylation assay in HPASMCs. As shown in Fig. 4.A, LETM1 was detected to be associated with the thiopropyl beads in the presence of hydroxylamine compared to the negative control of Tris·HCl. The result indicated that palmitoylation did exist in LETM1 and the level of palmitoylation was very high, which was consistent with the results of MS. In addition, we also detected the palmitoylation of LETM1 in Hela cells by the same assay and obtained the same result as that in HPASMCs (Fig. 4.B).

Next, we identified the specific palmitoylated cysteine residues of LETM1. As shown in Fig. 4.C and 5.E, the mitochondrial target sequence (1aa-114aa) was located at the N-terminal, TM1 (206aa-228aa) and TM2 (413aa-421aa) were the transmembrane domains of LETM1, and the N-terminal and C-terminal were located in the mitochondrial matrix. The domain analysis of LETM1 was original from Gayathri et al [28].

There was only cysteine 552 at the C-terminus of LETM1, which was conserved among human, mouse, rat and zebrafish (Fig. 4.D). To confirm whether the cysteine 552 was required for LETM1 palmitoylation, we generated a mutant form of LETM1 in which the cysteine (C) site was mutated to alanine (A). Then, we over-expressed LETM1-Flag and LETM1C552A-Flag in Hela cells and performed S-palmitoylation assay respectively. As shown in Figure 4E, the palmitoylation level of LETM1C552A was lower than that of wild type LETM1.

Therefore, we conclude that LETM1 is palmitoylated at cysteine 552, which is located in the cytoplasmic domain at C-terminus.

cGMP inhibits the palmitoylation of LETM1 and the activity of AKT.

Since LETM1 was palmitoylated, we tested whether cGMP regulated the palmitoylation level of LETM1. As shown in Figure 5.A, 8-Br-cGMP inhibited the palmitoylation level of LETM1 dramatically compared to the negative control, indicating that cGMP was a negative regulator of the palmitoylation of LETM1. Based on the result, we wondered how cGMP regulated the downstream biological processes by inhibiting the palmitoylation of LETM1. The PPI network analysis of LETM1 showed that LETM1 engaged in mitochondrial transport, which was involved in two aspects (Fig. 5.B). One was to regulate mitochondrial calcium ion transport (red marks) by cooperating with MICU, MICU1, MICU2, SLC8B1, etc. The other was to regulate AKT activity via the cooperation with THEM4 (acyl-coenzyme A thioesterase) (Fig. 5.B). According to the analysis, we carried out co-immunoprecipitation experiments in HPASMCs and found that endogenous LETM1 and THEM4 could be immunoprecipitated in HPASMCs (Fig. 5.C). Considering that THEM4 was involved in regulating the activity of AKT [29], we tested whether cGMP affected the activity of AKT in HPASMCs. After 8-Br-cGMP treatment, the phosphorylation level of AKT was significantly downregulated (Fig. 5.D). The result indicates that cGMP is the negative regulator of AKT activity. Taken together, we conclude that cGMP inversely regulates the palmitoylation of LETM1 and downregulates the activity of AKT.

Discussion

Palmitoylation at cytoplasmic domains is essential for transmembrane transport proteins, which is involved in membrane anchoring for regulating protein-protein interactions, the function of the proximate transmembrane domains, and so on [11]. Among the palmitoylated transmembrane transport proteins which have been studied so far, the majority of them localize to the plasma membrane, such as ion channels [12, 13], while almost rarely in the mitochondrial membrane. Previous studies found that LETM1, a protein in the inner mitochondrial membrane, participates in a variety of cellular functions, such as mitochondrial metabolism and cellular signaling [28].

THEM4 inhibits the phosphorylation of AKT by binding its hydrophobic motif [29] and interacts with LETM1 to regulate glucose metabolism in liver cells from obese and high fat diet-fed mice [30]. However, there is no evidence to explain that how LETM1 regulates the AKT activity through THEM4, except that the LETM1 expression is inversely correlated with the THEM4 expression and positively correlated with the AKT activity [30]. In this study, we found that LETM1 was palmitoylated by the palmitoylome with click-chemistry-probes (Fig. 1). More importantly, cGMP could inhibit the palmitoylation level of LETM1 in HPASMCs (Fig. 5.A). Our study also found that endogenous LETM1 could be immunoprecipitated with THEM4 in HPASMCs (Fig. 5.C). Meanwhile, we found that cGMP could inhibit the palmitoylation level of LETM1 and downregulate the AKT activity in HPASMCs (Fig. 5.A and 5.D). Based on the results, we speculated that LETM1 prevented THEM4 exporting from mitochondria to cytoplasm through palmitoylation, while cGMP promoted the release of THEM4 to inhibit the AKT activity by inhibiting the palmitoylation of LETM1 (Fig. 5.E). However, in view of the fact that the primary HPASMCs were difficult to obtain and varied greatly among individuals, we need to conduct in vivo experiments in mouse models to verify the speculation. In addition, we also obtained other palmitoylated proteins involved in mitochondrial transport and would pay attention to them in the future.

Conclusion

In this work, we found that LETM1 was a palmitoylated protein in the inner mitochondrial membrane. Moreover, cGMP could inhibit the palmitoylation level of LETM1 and suppress the AKT activity in HPASMCs. Our studies not only explored the nature of the interaction between LETM1 and THEM4 but also unraveled the new advance in understanding of cGMP signaling pathway in PAH. The palmitoylated site of LETM1 may provide new directions for the development of novel therapies for PAH. Moreover, the palmitoylated level of LETM1 may represent a new biomarker for analyzing prognosis.

Declarations

Statement of Ethics

This study was approved by the Beijing Hospital Ethics Committee (BHEC) with the registration No. of 2017BJYYEC-108-05.

Conflict of Interest Statement

None.

Authors' contributions

Wenqing Li: conceptualization, data curation, formal analysis, methodology, software and writing-original draft. Wei Huang: data curation, methodology. Ran Miao: methodology, investigation. Lihui Zou: funding acquisition, supervision. Fei Xiao: software, funding acquisition, writing-review & editing. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data Availability Statement

All the data are available and can be found.

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Figures

Figure 1

Experimental strategy for the identification of the palmitoylome using click chemistry reaction in HPASMCs. Cultured cells were incubated with Alk-C16, an alkynyl-palmitic acid. The Alk-C16-conjugated proteins were bound to the azide agarose beads by click chemistry reaction. The proteins were trypsin digested and subjected to LC-MS/MS and palmitoylome analysis.

Figure 2

Enriched terms of the transport-related proteins of palmitoylome in HPASMCs.

Functional enrichment of 1803 palmitoylated proteins were performed by Metascape web-based platform and the transport protein clusters were screened. Enriched terms with a p -value < 0.01 , a minimum count of 3, and an enrichment factor > 1.5 are collected and grouped into clusters based on their membership similarities. "Log10 (P)" is the p -value in log base 10.

Figure 3

Protein-protein interaction network constructed with the small molecules transport proteins. Nodes represent proteins, and the number of nodes is 52. Edges represent protein-protein associations, and the number of edges is 113. Line thickness indicates the strength of data support. Average local clustering coefficient: 0.524, PPI enrichment p -value: $<1.0e-16$. Different colors of the nodes represent different GO terms.

Figure 4

LETM1 is a novel palmitoylated protein at C-terminus. LETM1 palmitoylation was determined using an S-palmitoylation assay in HPASMCs (A) and in Hela cells (B). (C) Domain schematic of LETM1 including MTS (mitochondrial targeting sequence), TM1 (conserved transmembrane domain 1), TM2 (putative transmembrane domain 2), EF1, EF2 (non-canonical Ca^{2+} -binding EF-hand domains). (D) Alignment of LETM1 amino acid sequence with its homolog for conserved cysteines at C-terminus. Alignment of the LETM1 sequence in human (Uniprot accession number: O95202) with that of LETM1 in mouse (Uniprot accession number: Q9Z2I0), LETM1 in Rat (Uniprot accession number: Q5XIN6), LETM1 in Zebrafish (Uniprot accession number: Q1LY46). Highly conserved residues are shown in black. (E) LETM1 was palmitoylated at cysteine 552. Hela cells were transiently expressed LETM1-Flag (wild type) or LETM1^{C552A}-Flag. The cell lysates were immunoprecipitated using protein A/G resin preloaded with Flag antibody, and then subjected to S-palmitoylation assay to measure palmitoylation level of LETM1 and its mutant. HAM and Palm are abbreviations for hydroxylamine and palmitoylation, respectively.

A

B

● Mitochondrial calcium ion transmembrane transport
● Mitochondrial transport

C

E

D

Figure 5

cGMP inhibits the palmitoylation of LETM1 and suppresses the activity of AKT. (A) 8-Br-cGMP inhibited the palmitoylation of LETM1 in HPASMCs determined by S-palmitoylation assay. HPASMCs were treated with H₂O (as a control) or 8-Br-cGMP. Cell lysates were immunoprecipitated using protein A/G resin preloaded with LETM1 antibody and then subjected to S-palmitoylation assay. HAM and Palm are abbreviations for hydroxylamine and palmitoylation, respectively. (B) The biological process of LETM1 was analyzed by PPI combined with GO enrichment. (C) Endogenous LETM1 and THEM4 were immunoprecipitated in HPASMCs. Cell lysates were immunoprecipitated using protein A/G resin

preloaded with LETM1 antibody. Western blots were performed to analyze the presence of THEM4. (D) 8-Br-cGMP inhibited the activity of AKT. HPASMCs were treated with H₂O (as a control) or 8-Br- cGMP, and then lysed for analyzing the presence of pS473-AKT and AKT by western blot. (E) Mechanistic summary of the effects of cGMP on LETM1 palmitoylation and AKT activity. The dotted lines indicate that the function of LETM1 is unknown.

Supplementary Files

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- [TableS1.docx](#)
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