Dysregulation of very long chain acyl-CoA dehydrogenase coupled with lipid peroxidation

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Abstract

Kabuyama Y, Suzuki T, Nakazawa N, Yamaki J, Homma MK, Homma Y. Dysregulation of very long chain acyl-CoA dehydrogenase coupled with lipid peroxidation. Am J Physiol Cell Physiol 298: C107–C113, 2010. First published November 4, 2009; doi:10.1152/ajpcell.00231.2009.—Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disease of unknown etiology. We previously revealed increased oxidative stress and high expression of antioxidant proteins in culture cell lines established from lesional lung tissues with IPF (Kabuyama Y, Oshima K, Kitamura T, Homma M, Yamaki J, Munakata M, Homma Y. Genes Cells 12: 1235–1244, 2007). In this study, we show that IPF cells contain high levels of free cholesterol and its peroxidized form as compared with normal TIG lung fibroblasts, suggesting that radical oxygen species (ROS) are generated within specific organelles. To understand the molecular basis underlying the generation of ROS in IPF cells, we performed proteomic analysis of mitochondrial proteins from TIG and IPF cells. This analysis shows that the phosphorylation of Ser586 of very long chain acyl-CoA dehydrogenase (VLCAD) is significantly reduced in IPF cells. Similar results are obtained from immunoblotting with an anti-pS586 antibody. Kinase activity toward a peptide containing Ser586 from IPF cells is significantly lower than that from TIG cells. Furthermore, a phosphorylation-negative mutant (S586A) VLCAD shows reduced electron transfer activity and a strong dominant-negative effect on fatty acid β-oxidation. The ectopic expression of the S586A mutant induced human embryonic kidney (HEK) 293 cells to produce significantly high amounts of oxidized lipids and hydrogen peroxide. HEK293 cells expressing the S586A mutant exhibit a reduction in cell growth and an enhancement in apoptosis. These results suggest a novel regulatory mechanism for homeostatic VLCAD activity, whose dysregulation might be involved in the production of oxidative stress and in the pathogenesis of IPF.

β-oxidation; phosphorylation; protein kinase A; reactive oxygen species; apoptosis

Mitochondrial fatty acid β-oxidation is one of the main energy-producing metabolic pathways in eukaryotes. The rate-limiting step, the α-, β-dehydrogenation of a substrate, is catalyzed by enzymes belonging to the family of acyl-CoA dehydrogenases with distinct but overlapping chain-length specificities (7). Very long chain fatty acid dehydrogenase (VLCAD) has activity mainly toward CoA-esters of fatty acids with 16–24 carbons in length (7). In addition, VLCAD has been shown to catalyze the major part of palmitoyl-CoA dehydrogenation in many human tissues and cultured cells (2), indicating its central role in the catabolism of long-chain fatty acids. This is clearly reflected by the severe clinical symptoms caused by VLCAD deficiency, such as a high incidence of cardiomyopathy in childhood (8). So far, more than 60 pathogenic mutations have been identified, with clear correlations between the genotype and the disease severity (1). Biochemical studies including the determination of the crystal structure, as well as the elucidation of reaction mechanism of α-, β-dehydrogenation, have provided important information about how clinical phenotypes are induced by mutations in this gene (13, 16). However, little is known about the mechanism of VLCAD regulation at the level of posttranslational modification.

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown etiology characterized by accumulation and deposition of extracellular matrix that results in impaired pulmonary function (10). Key effectors in IPF are myofibroblasts functioning as the primary source of extracellular matrix proteins. Thus, the inhibition of myofibroblast differentiation/hyperproliferation may serve as an effective antifibrotic therapeutic option (17). On the other hand, recent studies have suggested an important role of radical oxygen species (ROS) in the pathogenesis of IPF. The protein expression patterns of IPF cells reveal an upregulation of biomarkers of oxidative stress and antioxidant proteins (14). This is further supported by the results of randomized clinical trials of treating IPF patients with N-acetylcysteine (NAC), a precursor of the major antioxidant glutathione. The data suggest that NAC has beneficial effects for patients with IPF (6). We previously reported that myofibroblasts isolated from patients with IPF show a significant elevation of ROS, resulting in lipid peroxidation and cell apoptosis (11). It has also been reported that myofibroblasts secrete hydrogen peroxide as a diffusible death signal for lung epithelial cells (24). These studies clearly suggest the function of myofibroblasts as ROS generators in fibrotic lesions. However, the precise mechanisms of ROS generation that lead to pathophysiologic phenotype remain elusive.

In this study, proteomics analysis of myofibroblasts was carried out to elucidate the mechanism of ROS generation in IPF. Our analysis reveals that the phosphorylation of VLCAD at Ser586 is inhibited in myofibroblasts, resulting in a significant loss of enzyme activity coupled with lipid peroxidation. Thus Ser586 represents a critical site for VLCAD activity, whose dysregulation might contribute to the progression of IPF and other oxidative-stress mediated diseases.

Materials and Methods

Cell culture and transfection. All experiments were carried out with the approval of the Fukushima Medical University Review Board. Normal human lung fibroblasts TIG7 and IPF myofibroblasts (LF1 and LF2) were cultured as described previously (11) and used for experiments before passage 5. Human embryonic kidney (HEK) 293 cells were maintained at 37°C in 5% CO2 in DMEM (Sigma) supplemented with 10% fetal bovine serum. Human retinal pigment epithelial (RPE) cells immortalized with telomerase (Clontech) were...
grown in DMEM-F-12 (Sigma) with 10% fetal bovine serum. Cell growth was assessed by counting cells, and apoptosis was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit according to the manufacturer’s recommended protocol (Cayman Chemical).

Cell treatment. For the treatment of LDL, TIG7 and LF cells were cultured for 24 h in serum-free medium containing LDL (Merck, Darmstadt, Germany) at a concentration of 50 μg/ml in the presence or absence of methyl-β-cyclodextrin (Sigma). These cells were used for determining the levels of cholesterol and peroxidized lipids. To confirm the involvement of PKA in the phosphorylation of VLCAD, HEK293 cells were treated with 100 μM dibutyl cyclic AMP (Dib-cAMP, Sigma) for 6 h, and cellular proteins were analyzed by two-dimensional gel electrophoresis (2D gel). Transfection was carried out using FuGENE 6 (Roche) at 50% confluency, and cells were harvested for experiments after culture for 2 days. Plasmid constructs were prepared as follows: The full-length VLCAD cDNA was PCR amplified from a human fetus brain cDNA library (Clontech) using primers 5'-GAATTCAGGCAGGCTCGATGCG-3' and 5'-GGGCCCCTGATGAGCCAATGTGGTTCG-3'. VLCAD was mutated at Ser586 with Ala (S586A) using the PrimeSTAR mutagenesis kit (Takara Bio, Shiga, Japan) and confirmed by BigDye sequencing (Applied Biosystems). Wild-type (WT) and S583A VLCAD were cloned into TOPO vector (Invitrogen) to generate C-FLAG-VLCAD and C-FLAG-S586A.

Preparation of a mitochondria-enriched fraction. Mitochondria were isolated from cultured cells by differential centrifugation. Cells were suspended and homogenized in a Potter’s glass homogenizer with H-Buffer (10 mM Tris · HCl, pH 7.5, 0.25 M sucrose), followed by centrifugation at 600 g for 5 min at 4°C. The supernatant was centrifuged at 8,000 g for 5 min and washed with H-Buffer, and the resulting pellet was resuspended in H-Buffer. The suspension was layered over a discontinuous sucrose gradient consisting of 1.0 M and 1.5 M sucrose interface, diluted with 5 volumes of 10 mM Tris · HCl at 26,000 g, and centrifuged for 1 h at 4°C. The mitochondria were collected from the 1.0 to 1.5 M sucrose interface, diluted with 5 volumes of 10 mM Tris · HCl, pH 7.5, and centrifuged at 25 min at 26,000 g at 4°C. The mitochondria were collected from the 1.0 to 1.5 M sucrose interface, diluted with 5 volumes of 10 mM Tris · HCl, pH 7.5, and centrifuged at 8,000 g for 5 min at 4°C. The pellet was suspended in 10 mM Tris · HCl, pH 7.5, and used for experiments.

2D gel and mass spectrometry. Proteins were extracted from the isolated mitochondria-enriched fraction and processed for isoelectric focusing as described previously (12) using IPG gel strips (pI 4–7 and 18 cm, GE Healthcare) and 10% SDS-PAGE. 2D gels were analyzed using a Melanie III Viewer (GeneBio) to detect protein spots. Responsive gels were visualized using methanol fixation (Silver stain MS kit, Wako, Tokyo, Japan). 2D gel images were analyzed using a Melanie III Viewer (GeneBio) to detect protein spots. Responsive gels were visualized using methanol fixation (Silver stain MS kit, Wako, Tokyo, Japan). 2D gel and mass spectrometry. Proteins were extracted from the isolated mitochondria-enriched fraction and processed for isoelectric focusing as described previously (12) using IPG gel strips (pI 4–7 and 6–11, 18 cm, GE Healthcare) and 10% SDS-PAGE. 2D gels were loaded with 30 μg of mitochondrial protein and were silver stained using methanol fixation (Silver stain MS kit, Wako, Tokyo, Japan). Gels were analyzed using a Melanie III Viewer (GeneBio) to detect changes in protein spot intensities on the gel images (12). Responsive proteins were identified as changing by more than twofold in each of the two biological replicate samples. Proteins were identified by in-gel digestion from wet gels using modified porcine trypsin (100 ng/digestion, Promega), and desalted on C18 ZipTips (Millipore). The resulting peptides were analyzed by tandem mass spectrometry using a Q-TOF electrospray ionization mass spectrometer (QSTAR, Applied Biosystems).

Immunoblotting analysis. Cellular or mitochondrial proteins were diluted in 50 mM Tris · HCl, pH 7.2, 2% SDS, 10 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate, and the protein amount was determined with a DC protein assay (Bio-Rad). Extracts (20 μg protein) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 and were incubated with primary antibody diluted with 5% bovine serum albumin (BSA) in TBS. Primary rabbit polyclonal antibodies were raised against synthetic peptides corresponding to residues 356–369 of human VLCAD, and phosphospecific antibodies were produced using a synthetic phosphopeptide corresponding to residues 582–591 of human VLCAD containing phosphoserine at residue 586 (CLSRARpSRLS). Anti-FLAG antibodies (Sigma) were also used as primary antibody. Blots were probed with goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Bio-Rad) and were visualized by enhanced chemiluminescence (ECL Western Blotting Systems, GE Healthcare).

VLCAD assay. Acyl-coenzyme A dehydrogenase activity was measured using the ferricenium ion as an electron acceptor (15). Briefly, assays were carried out at 25°C in 100 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 50 μM palmitoyl-CoA, 0.2 mM ferricenium hexafluorophosphate, and 10 μg isolated mitochondria. Enzyme activity was determined by following the decrease in absorbance at 300 nm upon the reduction of ferricenium ion. Mitochondrial fatty acid β-oxidation activity was measured by palmitoyl-CoA degradation to acetyl-CoA (18). The standard reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 35 μM 2,6-dichlorophenolindophenol, 1 mM NAC, 25 μM FAD, 0.2 mM NAD+, 50 μM CoA-SH, 0.2 mg/ml BSA, 50 μg isolated mitochondria, 1.6 mM phenazine methosulfate, and 32 μM palmitoyl-CoA (220,000 dpm) in the presence or absence of 1.6 mM phenazine methosulfate (PMS, primary electron acceptor) and 35 μM 2,6-dichlorophenolindophenol (DCP, secondary electron acceptor). After 10 min incubation at 30°C, the reaction was terminated by adding BSA (final 1 mg/ml) followed by perchloric acid (final 400 mM), and the samples were centrifuged. The supernatant was extracted with hexane and the radioactivity in the aqueous phase was measured. Specific activity was calculated by subtracting the values obtained from assay mixes in the absence of PMS and DCP from those in the presence of PMS and DCP.

In vitro kinase assay. An aliquot of mitochondria-rich fraction containing mitochondrial protein (~10 μg) was incubated with a substrate peptide (CLSRASRSLSE) at a final concentration of 200 μg/ml or 0.2 μg of glutathione S-transferase (GST) fusion protein in a phosphorylation buffer consisting of 20 mM Tris · HCl, pH 7.5, 0.1 M β-glycerophosphate, 0.1 M dithiothreitol, 0.1 M MgCl2, 0.1 M ATP, and 74 kBq32P-ATP in a total volume of 30 μl. A GST fusion protein containing the COOH-terminal 258 amino acids of VLCAD protein (GST-C-WT) was expressed in Escherichia coli and purified with glutathione Sepharose 4B (GE Healthcare). For the phosphorylation of the peptide, the phosphorylation products were collected onto a P81 filter (Whatman), which was thoroughly washed with 175 mM phosphate acid and used for the determination of radioactivity by scintillation counting. Phosphorylation of the GST fusion protein was assessed by SDS-PAGE followed by autoradiography.

Indirect immunofluorescence. Indirect immunofluorescence was carried out as described previously (11). Anti-FLAG mouse primary antibodies (Sigma) were followed with donkey anti-mouse IgG-AlexaFluor 488/546 (Invitrogen) and visualized with a fluorescence microscope system (Axiovert, Zeiss). Intracellular cholesterol was visualized by probing cells with 10 μg/ml filipin III (Sigma). To visualize the peroxidized lipids, cells were labeled with 100 μM diphenylpyrenylphosphine (DPPP, Doshindo, Tokyo, Japan) for 12 h. Mitochondria were visualized by MitoTracker Red (Invitrogen).

Measurement of ROS. Hydrogen peroxide and lipid hydroperoxides were detected using dichlorofluorescin diacetate (DCFDA) and DPPP, respectively. Labeling and measurements of fluorescence intensities were carried out as described previously (11).

Thin-layer chromatography. Total lipids were extracted by the Bligh and Dyer method (3). Briefly, 100 μl of cell suspension was mixed with 250 μl MeOH and 125 μl CHCl3. To this mixture was added 125 μl CHCl3 and 125 μl PBS, and the samples were centrifuged to separate the aqueous and organic solvent phases. The lower organic phase was subjected to thin-layer chromatography (TLC; silica gel 60, Merck, hexan:diethyl ether:acetic acid, 80:20:1). The separated lipids were visualized by 20% sulfuric acid. The silica gel in the location of the cholesterol, cholesterol ester, and phospholipids fractions was scraped off from another TLC plate developed in parallel and incubated with 100 μM DPPP for 2 h at room temperature. Reactivity of each fraction with DPPP was determined by fluorescence.
RESULTS

Peroxidation of cholesterol in IPF myofibroblasts. We previously reported that lipid peroxidation is enhanced in myofibroblasts isolated from patients with IPF (LF cells) (11). This was confirmed by cell staining with filipin III, a fluorescent probe that specifically binds to cholesterol molecules (23). IPF myofibroblasts showed a punctate staining pattern, in clear contrast to TIG7, normal pulmonary fibroblasts, which showed only faint staining (Fig. 1A). Interestingly, a similar punctate staining pattern was detected in LF cells by DPPP, a molecular probe that becomes fluorescent upon oxidation by lipid hydroperoxides. This punctate staining by DPPP was detected in LF cells treated with LDL, while serum-starved cells did not show any significant staining, indicating selective peroxidation of LDL-derived lipids (Fig. 1B). In addition, DPPP staining was not detected in IPF myofibroblasts treated with methyl-β-cyclodextrin, which selectively extracts cholesterol from the plasma membrane. To verify cholesterol peroxidation, cholesterol was separated from cholesterol ester and phospholipids by TLC, and then reacted with DPPP. This analysis confirmed cholesterol as a major peroxidized lipid in IPF myofibroblasts (Fig. 1C). Together, these results suggest that LDL-cholesterol absorbed by myofibroblasts is a main source of lipid hydroperoxides and accumulates in punctate structures.

Proteomic analysis of mitochondrial proteins. The punctate distribution of oxidized LDL-derived cholesterol suggests that ROS are generated within specific organelles. This led us to analyze protein expression profiles in mitochondria, an organelle that functions as a major source of ROS. Proteomic analysis of mitochondrial proteins derived from TIG and IPF cells showed major changes in VLCAD. Proteomic analysis of mitochondrial proteins derived from TIG and IPF cells showed major changes in VLCAD, which appeared in both acidic and basic forms in normal mitochondria but mainly in the basic form in mitochondria from IPF cells (Fig. 2A). The disappearance of the acidic form of VLCAD is consistent with covalent modifications, such as phosphorylation. To examine whether the acidic spot represents a phosphorylated form of VLCAD, FLAG-tagged protein was expressed in HEK293 cells and then was treated with Dib-cAMP, an activator of protein kinase A. Figure 2B shows that Dib-cAMP significantly upregulates the amount of the acidic form of VLCAD,
suggesting the phosphorylation-mediated regulation of VLCAD. Analysis of the primary sequence of VLCAD using NetPhos 2.0 (4) suggested Ser586 as a possible site for PKA phosphorylation. As shown in Fig. 2C, an in vitro kinase assay carried out using PKA and a GST fusion protein containing the COOH-terminal 258 amino acids showed the protein to be efficiently phosphorylated in a time-dependent manner. Similar results were obtained using a synthetic peptide containing Ser586 and residues 581–591 as substrate. Mitochondrial lysates derived from both normal TIG and IPF cells phosphorylated this peptide (Fig. 2D), although a significant reduction is observed with the IPF lysates. This might be associated with our finding, shown in Fig. 2A, that the acidic form of VLCAD disappears from IPF cells.

**Phosphorylation of Ser586 is essential for VLCAD function.** To confirm the phosphorylation of VLCAD in vivo, we raised antibodies against synthetic phosphopeptide containing phosphoserine-586 (pSer586) and the surrounding sequence. Immunoblotting results using anti-PKAs antibody are shown. The experiment (n = 3) was repeated four times, and representative results are shown. Statistical significance of the difference between TIG and LF2 was evaluated by Student’s t-test; data are expressed as means and SE (*P < 0.05; **P < 0.01).

IPF cells were examined with this antibody, an immunoreactive band was observed in TIG, but not in IPF cells, confirming that the phosphorylation status of Ser586 is modulated in IPF cells (Fig. 3B). Figure 3A also shows that PKA is involved in the phosphorylation of Ser586 since the anti-PKA substrate antibody recognized wild-type VLCAD, but not S586A. Indirect immunofluorescence using anti-FLAG monoclonal antibody showed that both C-FLAG-WT and C-FLAG-S586A localize in mitochondria (Fig. 3C), suggesting that Ser586 phosphorylation might regulate enzyme activity rather than cellular localization. Next, we assessed whether Ser586 phosphorylation is essential for VLCAD activity. To examine the acyl-CoA dehydrogenase activity itself, the electron transfer activity of isolated mitochondria was measured by a ferricenium dye assay (15) using HEK293 cells overexpressing either C-FLAG-WT or C-FLAG-S586A. As shown in Fig. 4A, the expression of wild-type VLCAD significantly upregulated electron transfer activity, clearly demonstrating an enhancement of dehydrogenase activity. In contrast, the S586A mutant showed a significant reduction in electron transfer activity. Similar results were obtained in experiments to examine fatty acid β-oxidation activity (Fig. 4B). In particular, the S586A mutant exhibited a strong dominant-negative effect on fatty

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**Fig. 2. Proteomic identification of very long chain acyl-CoA dehydrogenase (VLCAD) phosphorylation. A:** two-dimensional (2D) gel images showing VLCAD changes in IPF myofibroblasts. Arrowheads represent protein spots identified as VLCAD by mass spectrometry. **B:** 2D gel images showing VLCAD phosphorylation by PKA. Human embryonic kidney (HEK) 293 cells expressing C-FLAG-WT were treated with dibutyryl-cAMP (Dib-cAMP), an activator of PKA. C-FLAG-WT was recovered with M2 affinity gel and analyzed on 2D gel. Arrowheads represent VLCAD spots that migrate with exactly the same pI as detected in A. **C:** phosphorylation of GST-C-WT by PKA. An aliquot of GST-C-WT (0.2 µg protein) was reacted with 0.1 units PKA and subjected to autoradiography. **D:** in vitro kinase assay was carried out using mitochondrial lysates derived from normal TIG and LF2 cells and a peptide containing serine-586 (Ser586) and surrounding sequence as enzyme sources and substrate, respectively. Immunoblotting results using anti-PKA antibody are shown. The experiment (n = 3) was repeated four times, and representative results are shown. Statistical significance of the difference between TIG and LF2 was evaluated by Student’s t-test; data are expressed as means and SE (*P < 0.05; **P < 0.01).
acyl β-oxidation. These results strongly suggest that the phosphorylation of Ser586 is essential for homeostatic VLCAD activity and that the dysregulation of this phosphorylation significantly affects fatty acid catabolism.

**S586A mutant causes lipid peroxidation and cell growth defect.** The above results indicate that the dysregulation of VLCAD activity by the expression of the S586A mutant might be due to a defect in electron transfer from VLCAD to an acceptor, which would cause electron leakage resulting in the generation of ROS. Thus, we examined ROS generation in cells expressing wild-type VLCAD and S586A. As expected, S586A expression caused a significant enhancement in amounts of lipid peroxides and hydrogen peroxide (Fig. 5, A and B). Peroxidized lipids accumulated in punctate structures (Fig. 5C) resembling the staining pattern detected in IPF myofibroblasts, suggesting that dysregulation of VLCAD could be a causal factor for the pathophysiologic phenotype of IPF. We then analyzed viability and apoptosis of HEK293 cells expressing wild-type VLCAD or the S586A mutant. As shown in Fig. 6, cells expressing the S586A mutant showed a decreased number of viable cells (Fig. 6A) with a significant increase in cell apoptosis (Fig. 6B). These data clearly suggest that the phosphorylation of Ser586 plays a key role in regulating the redox status of VLCAD, whose dysregulation is coupled to growth inhibition and cell apoptosis.

**DISCUSSION**

In this study, we demonstrate that VLCAD, a rate-limiting enzyme in fatty acid β-oxidation, is regulated by phosphorylation at Ser586 (Fig. 3) and that the suppression of this phosphorylation causes a perturbation in its enzyme activity (Fig. 4) and the generation of ROS (Fig. 5), leading to an accumulation of lipid peroxides in intracellular aggregates. Indeed, we also observed an accumulation of stearic acid, a VLCAD substrate in the fatty acid composition of IPF cells (Fig. 4) and the generation of ROS (Fig. 5), suggesting that dysregulation of VLCAD could be a causal factor for ROS generation and its biological significance remain elusive. Our results reveal phosphorylation of serine-586 is essential for VLCAD activity and that the suppression of this phosphorylation significantly affects fatty acid catabolism.

**Fig. 3. Determination of Ser586 phosphorylation.** To confirm the phosphorylation of VLCAD in vivo, we raised antibodies against a synthetic phosphopeptide containing phosphoserine-586 (pSer586) and its surrounding sequence. A: extracts of the mitochondria-enriched fraction from HEK293 cells overexpressing either the C-FLAG-WT or C-FLAG-S586A were tested for reactivity with an anti-pSer586 antibody. This antibody recognized only wild-type VLCAD, and not S586A mutant. Anti-PKA substrate antibody recognized wild-type VLCAD, but not S586A, suggesting an involvement of PKA in the phosphorylation of VLCAD. B: anti-pSer586 antibody recognized VLCAD in TIG cells. The reactivity was less in LF1 and LF2 cells, confirming the 2D gel results shown in Fig. 2A and the decreased phosphorylation in Fig. 2D. C: localization of the S586A mutant in mitochondria. Retinal pigment epithelial (RPE) cells expressing C-FLAG-S586A were probed with anti-FLAG antibodies (green) and Mito-Tracker (red).

**Fig. 4. Phosphorylation of serine-586 is essential for VLCAD activity.** A: acyl-CoA dehydrogenase activities of mitochondria isolated from HEK293 cells expressing C-FLAG-WT or C-FLAG-S586A using palmitoyl-CoA and ferricenium hexafluorophosphate as the substrate and electron acceptor, respectively. B: fatty acid β-oxidation activities of mitochondria isolated from HEK293 cells expressing C-FLAG-WT or C-FLAG-S586A. The expression levels were analyzed using anti-FLAG antibody. The experiment (n = 3) was repeated three times, and representative results are shown. Statistical significance between the wild-type and mutant against a sham was evaluated by Student’s t-test; data are expressed as means and SE (*P < 0.05; **P < 0.01).
tion-dependent VLCAD regulation, which may be directly coupled to ROS generation (Fig. 5). Thus, protein kinases responsible for the in vivo phosphorylation of VLCAD, which is associated with energy homeostasis, would have an important function in ROS generation. Indeed, we observed a significant reduction in kinase activity toward a substrate peptide containing Ser586 in IPF cells (Fig. 2D). The regulation of kinases, including PKA, may provide important information for understanding the regulatory mechanism of VLCAD.

Recent reports on the crystal structure of VLCAD (13, 16), together with mutation analyses of patients with VLCAD deficiency (1, 2, 8), have advanced our understanding of the regulatory mechanism of VLCAD. Ser586 locates at the COOH-terminal noncatalytic domain of VLCAD. This COOH-terminal domain is unique to VLCAD and is not detected in other types of acyl CoA dehydrogenases (7). Also, this domain may not determine substrate specificity (16). Thus, the COOH-terminal domain may have another function, such as interaction with regulatory molecules. In a recent study, it was proposed that residues 485–519 function as a membrane binding domain (16), and the clinical mutation S583W shows defects in membrane-binding activity, supporting a regulatory function of the COOH-terminal domain (20). Interestingly, Ser586 is somewhat distal to the membrane to function as a direct membrane-binding site (16). In addition, our results show that the defect in VLCAD activity is caused by blocking the efficient electron transfer from VLCAD to an electron donor. Together, we assume that Ser586 functions as a key residue for interaction with other regulatory proteins, such as electron transfer flavoprotein, a common electron donor for acyl-CoA dehydrogenases (9). Further analysis is required to address this conceptual idea.

The present study demonstrates that LDL-derived lipids might be the primary targets of VLCAD-induced peroxidation. Recently, the intracellular metabolic/transport pathway of LDL-derived lipids received much attention mainly because of the finding that defects in this pathway lead to Nieman-Pick type C disease (NPC) (19). The biochemical and cell biological features of NPC include an accumulation of cholesterol in late endosomes/lysosomes, and a lack of responsiveness to exoge-

Fig. 5. Enhanced reactive oxygen species (ROS) generation in the S586A mutant. A and B: the levels of lipid hydroperoxides (A) and hydrogen peroxide (B) in HEK293 cells expressing C-FLAG-WT or C-FLAG-S586A were measured using the fluorescence probes dichlorofluorescin diacetate (DCFDA) and DPPP. The experiment (n = 3) was repeated three times, and representative results are shown. Statistical significance of the difference between the wild-type and mutant against a sham was evaluated by Student’s t-test; data are expressed as means and SE (**P < 0.01). C: DPPP staining of RPE cells expressing C-FLAG-WT or C-FLAG-S586A. A punctate staining pattern similar to that of IPF myofibroblasts was observed, and the staining was enhanced in the mutant cells.

Fig. 6. Cell viability and apoptosis in the S586A mutant. A: HEK293 cells expressing C-FLAG-WT or C-FLAG-S586A were plated in 96-well plates at a density of 1 x 10^4 cells/well, and the cell number was determined after cultivating in the growth medium for 48 h. B: apoptosis was measured using a mitochondria membrane potential assay kit and is expressed as a ratio of fluorescence intensity of monomers to that of J-aggregates. The experiment (n = 3) was repeated three times, and representative results are shown. Statistical significance of the difference between wild-type and mutant against a sham was calculated by Student’s t-test; data are expressed as means and SE (*P < 0.05).
nous LDL that leads to an enhancement of cholesterol synthesis. Our preliminary results suggest that peroxidized cholesterol accumulates in lysosomes in IPF myofibroblasts (data not shown). Thus, the physiological features of NPC correlate surprisingly well with those of IPF myofibroblasts. Although the clinical symptoms of these diseases are distinct, a cross profiling of genes and proteins expressed in these cells would provide valuable information about cholesterol homeostasis. The intracellular traffic pathways of LDL-derived lipids are unlikely to involve the mitochondria (19). Thus, it is of interest that the peroxidation of spatially distal molecules, such as LDL-derived lipids, occurs in IPF mitochondria.

In summary, we demonstrate the phosphorylation status of Ser586 and its functional effects on VLCAD. This modification of VLCAD is identified as perturbed in IPF cells, and whether this posttranscriptional modification contributes to the pathophysiology of IPF and other oxidative stress-related diseases requires more direct investigation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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