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Degradation of acetoacetyl-CoA synthetase, a ketone body-utilizing enzyme, by legumain in the mouse kidney

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ABSTRACT

Acetoacetyl-CoA synthetase (AACS) is a ketone body-utilizing enzyme, which is responsible for the synthesis of cholesterol and fatty acids from ketone bodies in lipogenic tissues, such as the liver and adipocytes. To explore the possibility of AACS regulation at the protein-processing level, we investigated the proteolytic degradation of AACS. Western blot analysis showed that the 75.1 kDa AACS was cleaved to form a protein of approximately 55 kDa in the kidney, which has considerable high activity of legumain, a lysosomal asparaginyl endopeptidase. Co-expression of AACS and legumain in HEK 293 cells generated the 55 kDa product from AACS. Moreover, incubation of recombinant AACS with recombinant legumain resulted in the degradation of AACS. Knockdown of legumain with short-hairpin RNA against legumain using the hydrodynamics method led to a decrease in the 55 kDa band of AACS in mouse kidney. These results suggest that legumain is involved in the processing of AACS through the lysosomal degradation pathway in the kidney.

1. Introduction

Ketone bodies are an alternative energy source during fasting and diabetes [1]. The function of ketone bodies has been extensively studied in tissues such as brain and skeletal muscle, which utilize ketone bodies as an energy source [2,3]. We previously showed that ketone body metabolism is responsible for cholesterol homeostasis and adipogenesis [4,5], suggesting a significant role for ketone bodies as not only an energy source but also an important metabolic intermediate, which participates in several biological phenomena.

In the mitochondria, acetoacetate is activated by coenzyme A (CoA) transferase [succinyl-CoA:3-oxoacid CoA transferase (SCOT); EC 2.8.3.5] to acetoacetyl-CoA, which is then converted to acetyl-coA and introduced into the tricarboxylic acid (TCA) cycle for energy production. SCOT knockout mice exhibit marked hyperketonemia, showing the importance of the appropriate regulation of ketone body metabolism during the neonatal period [3,6].

In the cytosol, acetoacetate is converted to acetoacetyl-CoA by acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16) for the synthesis of cholesterol and fatty acids [7,8]. Our previous studies have shown that AACS has an important role for adipogenesis and cholesterol homeostasis and suggested that the regulation of AACS expression and activity is an important factor in lipid homeostasis [4,5]. Previous studies indicated that AACS is transcriptionally regulated by a peroxisome proliferator-activated receptor γ (PPARγ) and a CCAAT/enhancer binding protein α (C/EBPα) in adipocytes [9,10] and by sterol regulatory element-binding protein 2 (SREBP-2) in the liver and in Neuro-2a cells [4,11]. Additionally, regarding protein modification, AACS has been shown to be inactivated by acetylation of its lysine residue in *Streptomyces lividans* [12]. However, while the post-translational regulation and degradation systems for AACS in mammals are starting to be elucidated, much is still unknown.

Legumain (asparaginyl endopeptidase, AEP, EC 3.4.22.34) is in the C13 family of cysteine proteases and is conserved in plants and mammals [13,14]. Legumain is a proteolytic enzyme specific for the C-terminal side of asparagine, and high activity of this enzyme has been detected in the kidney and placenta and, to lesser extent, in the spleen and liver [15]. Legumain contributes to the maturation process of the lysosomal cysteine proteases, cathepsin H, B and L in the kidney cells [16]. Knockout mice of legumain represent disorders resembling hemophagocytic syndrome [17] in addition to kidney dysfunction [18]. A recent study showed that simvastatin, a HMG-CoA reductase, decreased glucose metabolism and atorvastatin-induced down-regulation of legumain mRNA. Moreover, decreased legumain activity and expression level were observed due to simvastatin [19,20]. These results suggest that

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the lysosomal degradation system via legumain is involved in glucose and cholesterol metabolism.

In the present study, we show that legumain is responsible for the limited proteolysis of AACS, which seems to be involved in the regulation of ketone body utilization.

2. Materials and methods

2.1. Mice

Male mice of the ddY strain (6 weeks old) were purchased from the Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All animal experiments were approved by the Hoshi University and followed the Animal Care Guidelines.

2.2. Mouse tissues and subcellular fractionation

Mouse tissues were homogenized in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 250 mM sucrose, 10 mM 2-mercaptoethanol (Nacarai tesque, Inc.), protease inhibitor (cOmplete, Mini, Roche Diagnostics) and phosphatase inhibitor (PhosSTOP, Roche Diagnostics). Six up-and-down strokes were used in a Braun Potter S homogenizer running at 1000 rpm. The homogenate was centrifuged (800*g*), and the pellet was discarded. The supernatant was centrifuged again at 12,000*g* for 10 min, and the resulting supernatant was collected. The pellet was resuspended with 200 μL homogenate buffer, and then three cycles of freezing and thawing were performed. The crude extracts were centrifuged at 12,000*g* for 10 min and the supernatant was collected for the quantification of legumain activity [15] and for the determination of protein concentration. The supernatant was further centrifuged at 100,000*g* for 1 h. The final supernatant was defined as the cytosolic fraction. The protein concentrations of the cell lysates were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.) [21].

2.3. Western blotting

For Western blotting, 7.5 μg of protein was separated using 7.5% SDS polyacrylamide gel electrophoresis (SDS–PAGE) [22] and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, GE Healthcare Life Sciences). The membranes were probed with specific antibodies: rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2500; SIGMA), rabbit anti-legumain (1:500; SantaCruz) and rabbit anti-AACS (1:2500) [23]. Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2500; GE Healthcare). The proteins were detected with Luminata Forte Western HRP substrate (Millipore) according to the manufacturer's instructions.

2.4. PCR-based cloning of mouse AACS and mouse legumain

The full-length cDNA encoding mouse AACS or legumain was amplified using the forward primer 5′-ATGTCCAAGCTGGCG CGGCT-3′ and the reverse primer 5′-TCAGAAGTCCTGCAGCTCAG-3′ from mouse liver cDNA using pfu ultra DNA polymerase (Stratagene). The PCR product was subcloned into *Hind* III/*Xba* I restriction sites of pcDNA3.1(+) vector (Invitrogen) and into *EcoR* I/*Sal* I restriction sites of pCMV-(DYKDDDDK)-C Vector (Clontech Laboratories, Inc.). The insert was sequenced and found to be identical with the mouse AACS [NCBI: RefSeq(Nucleotide) NM_030210] or legumain [NCBI: RefSeq(Nucleotide) NM_011175] sequence.

2.5. Transfection and legumain autoactivation

Human embryonic kidney (HEK) 293 cells were purchased from HSRRB (JCRB9068) and were maintained in minimal Eagle's medium supplemented with 10% fetal bovine serum. HEK 293 cells were transfected with legumain, AACS, and an empty vector using Lipofectamine LTX reagent and Plus reagent (Invitrogen) according to the manufacturer's protocol. After 72 h, the cells were harvested in TNE buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA and 1% Nonidet P-40]. Cells were disrupted by three cycles of freezing and thawing, and the resulting cell extract was centrifuged at 18,000*g* for 10 min at 4 °C. The supernatant was adjusted to pH 4.5 with 0.1 M citric acid containing 1 mM EDTA and 0.01% CHAPS and then incubated for 4 h at 30 °C in an orbital shaker (170 rpm) [24]. After protein quantification, the samples were subjected to Western blotting.

2.6. Recombinant purification and cleavage of AACS

The AACS-FLAG epitope-tagging vectors (6 μg) were transfected into the Lenti-X 293T Cell line (Clontech Laboratories, Inc.) using Lipofectamine LTX reagent and Plus reagent according to the manufacturer's protocol. After 3 days of culture, the cells were harvested in extraction buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1.5 mM EDTA, 1% Nonidet P-40, 10% glycerol and protease inhibitor (cOmplete ULTRA mini)], followed by four sonication cycles of 20 s on and 30 s off. Then, the lysate was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was collected for purification of mouse AACS.

For FLAG affinity purification, alpha-FLAG M2 beads (SIGMA) were washed twice with tris-buffer saline (TBS). Then, the beads were suspended in the supernatant, and rotated at 4 °C for overnight. The beads were packed into a chromatography column (SIGMA) and washed three times with TBS. The bound AACS-FLAG was eluted with 5 mL of 150 μg/mL FLAG peptide (SIGMA). The protein was concentrated by ultrafiltration through an Amicon Ultra 30 K device (Merck Millipore Ltd.) and then diluted with TBS.

The AACS-FLAG (500 ng) was reacted with each concentration of recombinant mouse legumain (C-terminal polyhistidine-tag, Sino Biological Inc.) in 20 μL of 0.1 M sodium citrate buffer (pH 5.0) containing 1 mM EDTA and 1 mM DTT for 2 h at 30 °C. The samples were added to Laemmli buffer and used for Western blotting.

2.7. Hydrodynamics

Short hairpin RNA (shRNA) specific for mouse legumain was generated according to the manufacturer's instructions. To knockdown mouse legumain, the sequence GGATGCCAGGCACGTCATT and AGATCATGTCTTCATTTAC were chosen for the shRNA target and inserted into pSINsi-DKII vector (Takara). To achieve legumain knockdown *in vivo*, we used hydrodynamics-based gene transduction as previously described [4,25] with minor modifications. Briefly, mice were injected (within 5 s) in the tail vein, using a 27-gauge needle, with 2 mL of TransIT-EE hydrodynamic delivery solution (Mirus Bio) containing 50 μg of plasmid encoding short-hairpin RNA against mouse legumain.

2.8. Legumain activity assay

Legumain activity was measured as previously described [18]. Tissue homogenates (10 μg) were incubated in 200 μL assay buffer [0.1 M citric acid (pH5.0) containing 1 mM EDTA and 1 mM dithiothreitol] including 10 μM AEP substrate [benzyloxycarbonyl-L-alanyl-L-alanyl-L-asparagine-4-methylcoumaryl-7-amide (Z-Ala-Ala-Asn-MCA, Peptide institute, INC.)]. Fluorescence readings

(excitation 360 nm, emission 460 nm) were taken in an ARVOTM X2 fluorescence plate reader (PerkinElmer Co., Ltd.).

3. Results

3.1. Tissue distribution of AACS protein

To evaluate the relative levels of AACS in the major organs, AACS protein levels in male ddY mice were examined by Western blot analysis of total protein extracts isolated from the skeletal muscle, cerebrum, cerebellum, spleen, liver, kidney, heart and lung. Consistent with our previous data [10], the brain, liver, kidney, and lung contained relatively high levels of AACS protein (Fig. 1). Among these organs, the kidney exhibited a somewhat distinctive electrophoretic profile, that is, the short form of an AACS band is detected in the kidney. These results suggest that AACS is post-translationally regulated, being cleaved at a specific site in the kidney.

3.2. Proteolytic degradation of AACS by legumain

Legumain is mainly expressed in the kidney and placenta and, to a lesser extent, in the liver and spleen [15], raising the possibility that AACS is cleaved by legumain and degraded by the lysosome pathway in the kidney. Therefore, we examined whether AACS is susceptible to legumain action. AACS and legumain were transiently expressed in HEK 293 cells. Cell lysates were incubated at pH 4.5, which is known to be the optimal pH for legumain activation. Overexpression of AACS alone caused no processing of this enzyme (Fig. 2A). On the other hand, co-transfection of AACS and legumain caused cleavage of AACS, mainly generating 55 and 45 kDa bands (arrows) in western blotting. Next, we investigated whether recombinant AACS-FLAG is degraded by recombinant legumain. The recombinant AACS was cleaved by the recombinant legumain, and degradation of AACS occurred in a dose-dependent manner (Fig. 2B and C). These results indicate that AACS is directly cleaved by legumain.

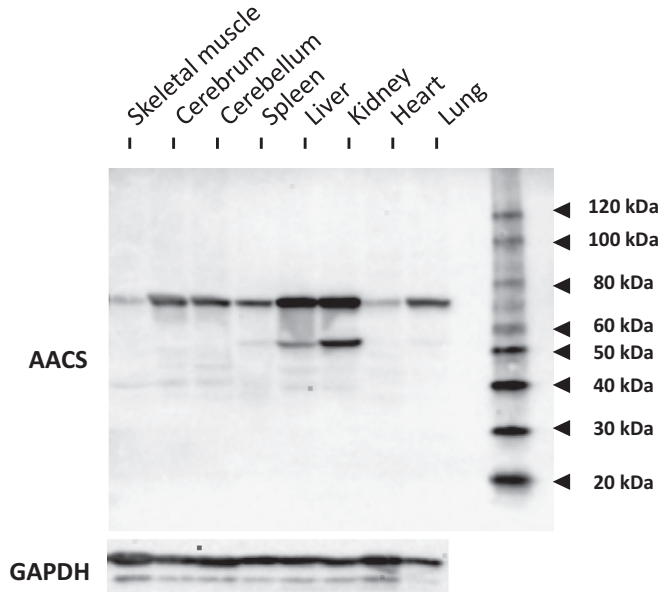

Fig. 1. Distribution of AACS protein in mouse tissues. Total protein was obtained from the skeletal muscle, cerebrum, cerebellum, spleen, liver, kidney, heart and lung, and subjected to Western blotting with specific AACS or GAPDH antibodies.

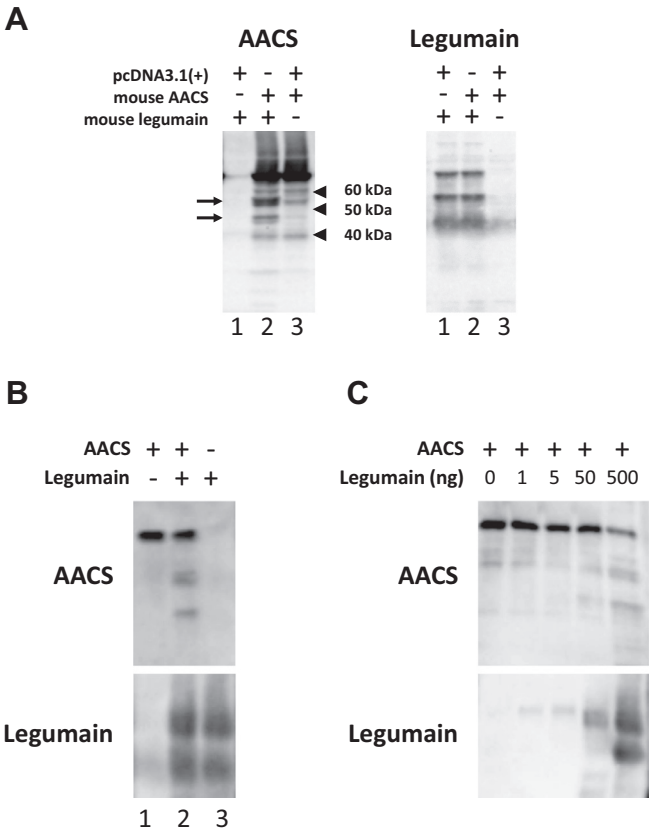

Fig. 2. Proteolytic degradation of AACS by legumain. (A) HEK 293 cells were transfected with the vector plasmid pcDNA3.1 (+) and mouse legumain construct (lane 1), mouse AACS construct and mouse legumain construct (lane 2), or the vector plasmid pcDNA3.1 (+) and mouse AACS construct (lane 3). Cell lysates were adjusted to pH 4.5 by the addition of 0.1 M citric acid containing 1 mM EDTA and 0.01% CHAPS, and incubated at 30 °C for 4 h. After incubation, each sample was subjected to Western blotting to detect AACS or legumain. The arrow indicates the cleaved form of AACS. (B) FLAG-AACS (500 ng) was reacted with or without 500 ng of recombinant mouse legumain (lane 1 or 2) in 0.1 M sodium citrate buffer (pH 5.0) containing 1 mM EDTA and 1 mM DTT at 30 °C for 2 h. Recombinant mouse legumain was also incubated alone in the same buffer (lane 3). Expression of AACS and legumain was analyzed by Western blotting with specific antibodies. (C) FLAG-AACS (500 ng) was reacted with the indicated concentration of the recombinant mouse legumain in the reaction buffer and then subjected to Western blotting.

3.3. Cleavage of AACS by legumain in vivo

Although AACS is cleaved by legumain *in vitro*, it is unclear whether AACS is cleaved by legumain in the kidney. Therefore, we carried out knockdown experiments with short hairpin RNA that targets mouse legumain (shLegumain) using the hydrodynamics method. Protein expression and the activity of legumain in the mouse kidney were significantly decreased by the shLegumain treatment (Fig. 3A and B). Suppression of legumain resulted in a decrease in the cleaved form of AACS protein, and an increase in the full length form of AACS protein (Fig. 3B–D). These data indicate that legumain is involved in the cleavage of AACS in the kidney, suggesting that AACS is degraded by the lysosomal pathway.

4. Discussion

Compared to the transcriptional regulation of ketone body utilization, its post-translational regulation remains almost unknown. In the present study, we presented the first evidence that suggests a possible involvement of a lysosomal degradation mechanism via legumain in the regulation of ketone body utilization, demonstrating limited proteolysis of AACS by legumain in mouse kidney.

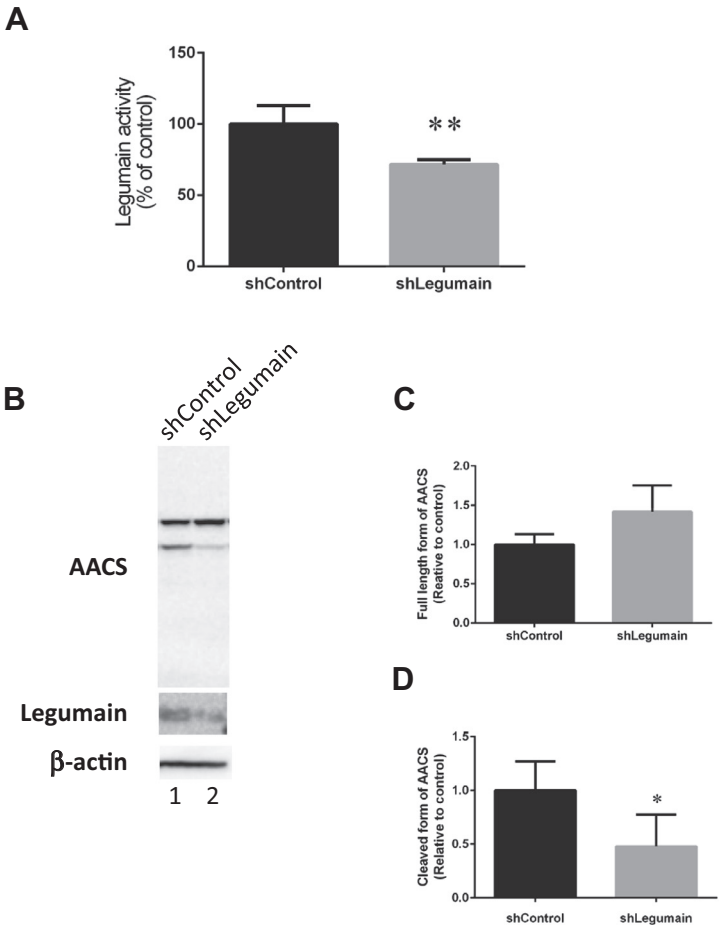

Fig. 3. Effect of shLegumain on cleavage of AACS in mouse kidney. (A) Mice were administered 50 μg of shLegumain vector via a tail-vein injection. After 48 h, the lysosomal fraction was extracted from the kidney. Legumain activity was then measured by fluorometric assays using Z-Ala-Ala-Asn-MCA as a substrate, as described in Section 2 ($n = 4$, $^{**}p < 0.01$). (B) Mice were administered 50 μg of shLegumain vector via a tail-vein injection. After 48 h, the cytosolic protein fraction was extracted from the kidney and subjected to Western blotting. (C and D) Intensity of full length and cleaved form of AACS was quantified ($n = 4$, $^{*}p < 0.05$).

A previous study showed that AACS mRNA is abundant in the kidney rather than in the liver [10]. However, in this present study, the expression level of AACS protein in the kidney is similar to that in the liver, and the short form of the AACS band is detected in the kidney (Fig. 1). These results suggest that translational control or post-translational modification may participate in the regulation of AACS protein. Legumain is an endoasparaginyl peptidase that is responsible for MHC degradation and dendritic cell maturation in the immune response and activation of the cathepsin families [26,27]. Legumain is mainly expressed in spleen and kidney, and it degrades various proteins in the lysosome. One of the main substrates for legumain is cathepsin, which is activated by legumain for the lysosomal function. In our results, AACS is also cleaved by legumain (Figs. 2 and 3), suggesting a functional role for this degradation pathway in AACS protein turnover. A previous study showed that the promoter region of AACS has a C/EBP family binding site with which C/EBP α and β directly interact in 3T3-L1 cells [10]. The C/EBP family is responsible for the immune response and the differentiation of T cells. These findings imply that AACS also has important roles in the immune response and in the differentiation of immune cells.

Legumain cleaves a C-terminal asparagine residue of specific motif [14]. AACS does not have such typical cleavage sequences but has characteristic sequences at position 500–503 (Asn-Asp-Glu-Asn) and 545–547 (Asn-Pro-Asn) at which cleavage would bring about the formation of a protein of approximately 55 kDa from the 75.1 kDa AACS. These results raise the possibility that cleavage of AACS by legumain is the initial step of AACS degradation. A previous study showed that legumain is essential for homeostasis and function of normal kidney proximal tubular cells (PTCs) [18,28]. Moreover, knockout mice of legumain develop hyperplasia of PTCs, and in AEP-null PTCs, EGF receptor levels are significantly higher, which would explain the hyperplasia. Previously, we showed that knockdown of AACS down-regulates the protein expression of synaptopodin in primary neurons [11]. Synaptopodin is associated with renal podocytes [29] and orchestrates actin organization and cell motility via the regulation of RhoA signaling [30]. AACS is also associated with the mevalonate pathway [4], which regulates the post-translational modification of RhoA [31]. Considering these findings, it is possible that AACS is associated with hyperplasia in the knockout mice of legumain in the kidney and tight regulation of AACS expression is necessary for these functions.

In summary, we found the cleaved form of AACS is abundant in the kidney. This cleavage was directly catalyzed by legumain, and knockdown of legumain in the kidney decreased the cleaved form of AACS and increased the intact form. Our findings may provide new insight into the relationship between ketone body metabolism and lysosomal function.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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