Klotho Protein Deficiency Leads to Overactivation of \( \mu \)-Calpain*

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The klotho mouse is an animal model that prematurely shows phenotypes resembling human aging. Here we report that in homozygotes for the klotho mutation \( kln^{-/-} \), \( \alpha \)II-spectrin is highly cleaved, even before the occurrence of aging symptoms such as calcification and arteriosclerosis. Because \( \alpha \)II-spectrin is susceptible to proteolysis by calpain, we examined the activation of calpain in \( kln^{-/-} \) mice. m-Calpain was not activated, but \( \mu \)-calpain was activated at an abnormally high level, and an endogenous inhibitor of calpain, calpastatin, was significantly decreased. Proteolysis of \( \alpha \)II-spectrin increased with decreasing level of Klotho protein. Similar phenomena were observed in normal aged mice. Our results indicate that the abnormal activation of calpain due to the decrease of Klotho protein leads to degradation of cytoskeletal elements such as \( \alpha \)II-spectrin. Such deterioration may trigger renal abnormalities in \( kln^{-/-} \) mice and aged mice, but Klotho protein may suppress these processes.

The klotho \( (kln^{-/-}) \) mouse shows multiple phenotypes resembling human aging caused by the mutation of a single gene (1). This mutation is caused by the insertion of ectopic DNA into the regulatory region of the klotho gene. The klotho gene encodes a type I membrane protein that is expressed predominantly in the kidney and brain. The extracellular domain of Klotho protein consists of two internal repeats that share sequence similarity to the \( \beta \)-glucosidases of both bacteria and plants (1, 2). As a result of a defect in klotho gene expression, the \( kln^{-/-} \) mouse exhibits multiple age-associated disorders, such as arteriosclerosis, osteoporosis, skin atrophy, pulmonary emphysema, short life span, and infertility. However, the mechanism by which the klotho gene product suppresses the aging phenomena has not been identified. Analysis of the pathophysiology of \( kln^{-/-} \) mice is expected to give clues not only to understanding the mechanisms of individual diseases associated with aging but also the relationship between these mechanisms during human aging.

Non-erythroid spectrin is a heterodimeric actin-binding protein that consists of \( \alpha \)II- and \( \beta \)II-spectrin and is usually found on the cytoplasmic side of the plasma membrane (3, 4). It is thought to participate in the establishment and maintenance of cell polarity, shape, and receptor distribution (5). Recently, it was proposed that spectrin retained and stabilized various proteins at specific regions on the cell surface (6–9). \( \alpha \)II-Spectrin has been shown to be cleaved by calpain and/or caspase during apoptosis and necrosis (10–13).

Calpain, a calcium-dependent cytosolic cysteine protease, is involved in many physiological and pathological processes (14–16). Calpain mediates proteolysis of various cellular proteins, including cytoskeletal proteins, and causes irreversible cell damage (10–13, 17, 18). Thus, calpain overactivation may contribute to the pathology of cerebral and cardiac ischemia, Alzheimer’s disease, arthritis, and cataaract formation (19, 20). Calpain has been shown to be regulated by both calcium ion and calpastatin (16). Two types of isozymic calpain, \( \mu \)-calpain, and m-calpain, are ubiquitously distributed in mammalian cells. The former is activated by micromolar concentrations of calcium and the latter is activated by millimolar concentrations of calcium. Calpastatin is an endogenous inhibitor specific for calpain, but is slowly degraded by calpain (21). Here, we report the cleavage of \( \alpha \)II-spectrin due to the continuous activation of \( \mu \)-calpain in \( kln^{-/-} \) mice. Furthermore, we also observe similar phenomena in normal aged mice.

**EXPERIMENTAL PROCEDURES**

Preparation of Mouse Tissue Extracts—Kidneys were obtained from 2- and 3-week-old \( kln^{-/-} \), \( kln^{-/-} \), and \( kln^{-/-} \) mice and from 4-week-old and 29-month-old C57BL/6 mice. Brain, lung, heart, liver, and kidney were obtained from 4-week-old and 8-week-old \( kln^{-/-} \) and \( kln^{-/-} \) mice. Tissue samples were homogenized with 9 volumes (weight/volume) of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose. After centrifugation at 900 \( \times g \) for 10 min, the supernatant was subjected to ultra centrifugation at 100,000 \( \times g \) for 1 h. The supernatants and precipitates were used as the cytosolic fraction and microsomal membrane fraction, respectively. Protein concentration was determined by BCA assay (Pierce). All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology. All efforts were made to minimize the number of animals used and their suffering.

**Amino Acid Sequencing of 280-kDa Protein—**Kidney microsomal fraction (250 \( \mu \)g) from 4-week-old \( kln^{-/-} \) and \( kln^{-/-} \) mice was subjected to SDS-PAGE under reducing conditions followed by staining with Coomassie Brilliant Blue R-250. A protein band of ~280 kDa was excised and treated with 0.1 \( \mu \)g of Achromobacter protease I (lysoedemoproteinase) at 37 °C for 12 h in 0.1 \( \times \) Tris-HCl, pH 9.0, containing 0.1% SDS and 1 \( \times \) EDTA (32). The peptides were separated on columns of DEAE-SPW (1 \( \times \) 20 mm; Tosoh, Tokyo, Japan) and CAPCELL PAK C18 UG120 (1 \( \times \) 100 mm; Shimadzu, Tokyo, Japan). Solvent A was 0.085% \( \approx \text{ trifluoroacetic acid in distilled water, and solvent B was 0.075%} \approx \text{ trifluoroacetic acid in 80% (v/v) acetonitrile. The peptides were eluted at a flow rate of 30 \( \mu \)l/min using a linear gradient of 1–60% solvent B. Selected peptides were subjected to Edman degradation.***
using a Procise 494 cLC protein sequencer (Applied Biosystems, Foster City, CA) and to matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Reflex MALDI-TOF (Bruker Daltonics, Billerica, MA) in linear mode using 2-mercaptobenzothiazole as a matrix.

**Antibodies**—Rabbit antibodies specific to the pre- and post-autolytic forms of $\mu$-calpain (anti-pre-$\mu$ and anti-post-$\mu$, respectively) were raised against synthetic peptides as described previously (23). Antibodies specific to the pre- and post-autolytic forms of m-calpain (anti-pre-m and anti-post-m, respectively) were produced using synthetic peptides corresponding to the N-terminal 21 residues (AGIAAKLAKDREAAEGLG-SHHP) of the intact form and the N-terminal 6 residues (KDREAA) of the autolytic form, respectively. A cysteine residue was added to the C terminus of each peptide so that the antigenic peptide could be conjugated to keyhole limpet hemocyanin. The entire amino acid sequence and the autolytic cleavage site of human m-calpain were obtained from previous reports (24, 25). Antibodies specific to the calpain-generated N- and C-terminal fragments of $\alpha_{17}$-spectrin (136 and 148 kDa, respectively) were produced by the peptide antigens QQQEVY (anti-BDP-136) and GAMPRD (anti-BDP-148), respectively (see Fig. 2). A cysteine residue was added to the N terminus of QQQE EVY peptide or was added to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site in mouse $\alpha_{17}$-spectrin by calpain were as determined by others previously (22, 26). An antibody against domain IV of human calpastatin was produced using a synthetic peptide corresponding to the cleavage site in mouse calpastatin and a cysteine residue added to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site of mouse calpastatin were as determined by others previously (22, 26). An antibody against domain IV of human calpastatin was produced using a synthetic peptide corresponding to the cleavage site in mouse calpastatin and a cysteine residue added to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site of mouse calpastatin were as determined by others previously (22, 26).

**Activation peptides**—Calpastatin was produced using a synthetic peptide corresponding to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site in mouse calpastatin were as determined by others previously (22, 26). An antibody against domain IV of human calpastatin was produced using a synthetic peptide corresponding to the cleavage site in mouse calpastatin and a cysteine residue added to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site of mouse calpastatin were as determined by others previously (22, 26). An antibody against domain IV of human calpastatin was produced using a synthetic peptide corresponding to the cleavage site in mouse calpastatin and a cysteine residue added to the C terminus of GAMPRD peptide. The amino acid sequence and the cleavage site of mouse calpastatin were as determined by others previously (22, 26).

**Results**

**Decrease of $\alpha_{17}$-Spectrin in the Kidney of $kl^{+/−}$ Mice**—To determine whether mouse homozygotes of the $klotho$ gene mutation ($kl^{+/−}$) have a different pattern of proteins in the kidney, we examined the kidney microsomal fractions from 4-week-old mice by SDS-PAGE. A band of about 280 kDa was found to be significantly weaker in $kl^{+/−}$ mice than in $kl^{+/+}$ mice (Fig. 1A). Similar results were obtained with five other $kl^{+/−}$ mice. The 280-kDa protein band was subjected to in-gel lysylendopeptidase digestion (22), and the sequences of two of the resulting peptides were determined to be LQTASDESYK and LAISAEVYK (GenBankTM accession number AAA41498).

A Western blot using an anti-$\alpha_{17}$-spectrin antibody (C-20) confirmed that the 280-kDa protein is $\alpha_{17}$-spectrin and that the reactivity of the antibody was dramatically decreased in $kl^{+/−}$ mice (Fig. 1B). The antibody also stained a 145-kDa band in $kl^{+/+}$ mice, but this band was below the detectable level in $kl^{+/−}$ mice (Fig. 1B). Because the anti-$\alpha_{17}$-spectrin antibody recognizes the C terminus of $\alpha_{17}$-spectrin, it is likely that the 145-kDa band is a C-terminal fragment of $\alpha_{17}$-spectrin. Although the kidney of 4-week-old $kl^{+/+}$ mice was not morphologically different from that of $kl^{+/−}$ mice, it did show a small amount of calcification (Fig. 1, C–F).

**Decrement of $\alpha_{17}$-Spectrin and Calpastatin with Increased Activation of $\mu$-Calpain—**$\alpha_{17}$-Spectrin was previously shown to be cleaved at a particular site by calpain (29), yielding 136- and 148-kDa fragments. To determine whether calpain is involved
showed that the expression level of Klotho protein affected the kl
were intermediate between those of kl
rather than a down-regulation of transcription. Pre-
and the inactive and active forms of m-calpain (pre- and post-
m-calpain). Pre-
was detected in kl’/’ mice but not in kl’/’ mice (Fig. 3C). Post-
was detected in kl’/’ mice but not in kl’/’ mice (Fig. 3D). Pre-m-calpain was detected in both kl’/’ and kl’/’ mice with no significant difference between them (Fig. 3E). Post-m-calpain was barely detected in either kl’/’ or kl’/’ mice (Fig. 3F). These results indicate that 
-m-calpain, but not m-calpain, was specifically activated in the kl’/’ kidney. Interestingly, calpastatin, which is an endoge-
nous inhibitor of calpain, was barely detected in 
/mice (Fig. 3B). These results indicated that 
-spectrin was degraded by calpain in the kidney of kl’/’ mice.

To determine which of the calpain isozymes were activated in the kl’/’ kidney, we made a Western blot of kidney cytosolic fractions using antibodies against four types of calpain: the inactive and active forms of 
-calpain (pre- and post-
-calpain) and the inactive and active forms of 
-calpain (pre- and post-
-calpain). Pre-
-calpain was detected in kl’/’ mice but not in kl’/’ mice (Fig. 3A). Post-
-calpain was detected in kl’/’ mice but not in kl’/’ mice (Fig. 3C). Pre-
-calpain was detected in both kl’/’ and kl’/’ mice with no significant difference between them (Fig. 3E). Post-
-calpain was barely detected in either kl’/’ or kl’/’ mice (Fig. 3F). These results indicate that 
-m-calpain, but not m-calpain, was specifically activated in the kl’/’ kidney. Interestingly, calpastatin, which is an endoge-
nous inhibitor of calpain, was barely detected in kl’/’ mice (Fig. 3B). The triplet bands at about 122 kDa in Fig. 3G are probably alternative splicing forms of calpastatin (30).

The expression levels of mRNAs of 
-spectrin (Fig. 3H) and 
-spectrin (Fig. 3I) were not different between kl’/’ and kl’/’ mice, which suggests that the decreases of calpastatin and 
-spectrin in kl’/’ mice were due to increased degradation rather than a down-regulation of transcription.

Activation of 
-Calpain Depends on Klotho Protein Level—To elucidate the relation between the amount of Klotho protein and the degree of 
-calpain activation, we examined the mouse heterozygotes for the klotho mutation (kl’/’). The expression level of Klotho protein in 2-week-old kl’/’ mice (Fig. 4A, lane 2), was approximately half that in 2-week-old kl’/’ mice (lane 1). A similar relation was found in 3-week-old mice (lanes 5 and 4, respectively). The levels of expression of pre-
-calpain, post-
-calpain, and 
-spectrin in 2-week-old kl’/’ mice (lane 2) were intermediate between those of kl’/’ mice (lane 1) and those of kl’/’ mice (lane 3). Similar results were obtained in 3-week-old mice (lanes 5, 4, and 6, respectively). These results showed that the expression level of Klotho protein affected the activation of 
-calpain and the amount of calpastatin (Fig. 4B).

To elucidate the process of calpain activation, calpastatin decrement, and 
-spectrin proteolysis, we examined mice that were less than 4 weeks old. In kl’/’ mice, pre-
-calpain and calpastatin were present at low levels at 2 weeks (Fig. 4A, lane 3) but were undetectable at 3 weeks (lane 6), while the amount of cleaved 
-spectrin was much higher at 3 weeks (lane 6) and 4 weeks than at 2 weeks (lane 3). In 2- and 3-week-old kl’/’ mice (lanes 2 and 5), the amount of cleaved 
-spectrin was much higher at 3 weeks (lane 5) than at 2 weeks (lane 2), while the levels of pre-
-calpain and calpastatin at 3 weeks were slightly less than those at 2 weeks. These findings suggest that:
1) pre-
-calpain and calpastatin were originally expressed in kl’/’ mouse kidney and that 
-calpain was gradually activated and calpastatin was gradually decreased during development, and
2) 
-spectrin was hardly cleaved in the presence of calpastatin, but intensive cleavage of 
-spectrin was observed after the complete disappearance of calpastatin (Fig. 4B). No nalcification was observed in 3-week-old kl’/’ mice (data not shown), indicating that degradation of 
-spectrin in the kidney of kl’/’ mice preceded the occurrence of any tissue damage.

Organ-specific Calpain Activation—The susceptibility and degree of proteolysis due to the klotho mutation varied among different organs. Changes in the lung of 4-week-old kl’/’ mice (Fig. 5) were similar to those observed in the kidney. The intensity of intact 
-spectrin drastically decreased and lower
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FIG. 5. Organ-specific differences in calpastatin, calpain, and \( \alpha_{II} \)-spectrin between \( kl^{+/+} \) and \( kl^{-/-} \) mice. Western blots of organ extracts of 4- and 8-week-old male mice with antibodies. Lanes W, \( kl^{+/+} \); lanes H, \( kl^{-/-} \). Arrowheads indicate the positions of each molecule. B, intensities of bands in A were measured by densitometric scanning using a densitometer and NIH Image 1.61/ppc software.

Fig. 4. Dependence of calpain, calpastatin, and \( \alpha_{II} \)-spectrin on Klotho protein. A, Western blots of kidney cytosolic and microsomal fractions of 2- and 3-week-old male mice using anti-Klotho antibody and other antibodies described in Fig. 3. Lanes 1 and 4, \( kl^{+/+} \); lanes 2 and 5, \( kl^{-/-} \); lanes 3 and 6, \( kl^{-/-} \). Arrowheads indicate the positions of each molecule. B, intensities of bands in A were measured by densitometric scanning using a densitometer and NIH Image 1.61/ppc software.

m-Calpain activity, in addition to being regulated by the calcium ion concentration, is usually also regulated by the binding of calpastatin (16). Thus, a deficiency of calpastatin may induce the overdestruction of substrates such as \( \alpha_{II} \)-spectrin by calpain. The mechanism by which Klotho protein might regulate \( \mu \)-calpain activity and calpastatin level in the kidney is unknown. However, it is possible that this regulation is mediated by nitric oxide (NO). NO has been shown to inhibit calpain-mediated proteolysis (32), and sys-
The expression of klotho function as a humoral factor. In support of this hypothesis, both the kidney and testis could improve systemic aging phenotypes in mice (1, 38). It is important to identify and characterize a gene expressed in the brain that the expression of klotho mRNA is closely related to aging processes. Recent studies revealed that the expression of klotho gene in the brain and testis could improve systemic aging phenotypes in klotho mice (1, 38). It is important to identify and characterize a target molecule (receptor) that is responsive to Klotho protein or its metabolites. Thus, it may be that the factor most responsible for an organism’s sensitivity to the klotho mutation is the density of such a receptor.

Our finding that normal aged mice show changes similar to those in klotho mice suggests that the decrease of Klotho protein is closely related to aging processes. Recent studies revealed that the expression of klotho gene was gradually reduced in the rat kidney during long term hypertension (39) and that calpastatin was gently degraded also in the kidney of hypertensive rats (40). Furthermore, humans with chronic renal failure commonly develop multiple complications resembling phenotypes observed in klotho mice (41–45), and the expression of klotho mRNA and the production of Klotho protein were severely reduced in these patients (46). Taken together, these results suggest that Klotho protein in the kidney protects the rat kidney from the development of age-related renal abnormalities and to prevent renal diseases in the future.

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