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Chaperone-Mediated Autophagy in the Kidney: The Road More Traveled

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Summary

Chaperone-mediated autophagy (CMA) is a lysosomal proteolytic pathway in which cytosolic substrate proteins contain specific chaperone recognition sequences required for degradation and are translocated directly across the lysosomal membrane for destruction. CMA proteolytic activity has a reciprocal relationship with macroautophagy: CMA is most active in cells in which macroautophagy is least active. Normal renal proximal tubular cells have low levels of macroautophagy, but high basal levels of CMA activity. CMA activity is regulated by starvation, growth factors, oxidative stress, lipids, aging, and retinoic acid signaling. The physiological consequences of changes in CMA activity depend on the substrate proteins present in a given cell type. In the proximal tubule, increased CMA results from protein or calorie starvation and from oxidative stress. Overactivity of CMA can be associated with tubular lysosomal pathology and certain cancers. Reduced CMA activity contributes to protein accumulation in renal tubular hypertrophy, but may contribute to oxidative tissue damage in diabetes and aging. Although there are more questions than answers about the role of high basal CMA activity, this remarkable feature of tubular protein metabolism appears to influence a variety of chronic diseases.

Keywords

Hypertrophy; diabetes; cancer; starvation

The word *autophagy* often is used to refer just to macroautophagy, while other lysosomal proteolytic pathways have received less attention. The common thread in autophagy is lysosomal or endosomal degradation of cellular proteins (referred to as *substrate proteins* or *cargo*), while the separate types of autophagy are defined by the mechanisms that the substrate proteins enter the lysosome.¹ These different autophagic mechanisms have unique physiologic relevance because different types of cargo are taken up by the different systems and there is different regulation of their uptake. Macroautophagy describes a particular mechanism that the cell uses to take up cytosolic proteins, lipids, or organelles in double-membrane vesicles, which then fuse with the lysosome.¹ Other forms of autophagy use entirely different mechanisms for transporting cargo proteins into endosomes or lysosomes.

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Microautophagy refers to internalization of proteins or organelles into lysosomes through invaginations in the lysosomal or endosomal membrane, forming a single vesicles inside the lysosome. Chaperones are now known to be involved in a variety of processes, including certain types of macroautophagy and microautophagy as well as proteasomal proteolysis, but chaperone-mediated autophagy (CMA) refers to the one-on-one targeting of proteins via a chaperone protein to the surface of the lysosome where the proteins are unfolded and translocated across the lysosomal membrane² (Fig. 1).

CMA has particular relevance to the renal proximal tubule because, in the basal state of the mature tubule, CMA is by far the predominant form of autophagy.³⁻⁵ Because of the selective nature of proteolysis and its targeting of longer half-life proteins, CMA has the power to influence specific protein levels and alter them in chronic disease.⁶ This review explores the relevance of this high level of CMA activity on proximal tubule physiology and pathophysiology. Although the data are sparse and the newer methods have not been applied to the kidney, the mechanisms and regulation of CMA provide insight into possible roles of CMA in renal health and disease.

MECHANISM OF CMA

Starvation selectively reduces the half-life of certain proteins, but not others.⁷ The search for selectivity mechanisms led to the discovery of CMA.⁸ Isolated lysosomes prepared by density gradient centrifugation using a particular supplier of metrizamide had the ability to directly degrade some proteins but not others.⁹ If lysosomes were prepared from serum-starved fibroblasts or livers of rabbits deprived of food for 24 hours then this destruction was increased.¹⁰ Electron microscopy did not show any vesicle formation, making it unlikely that a special form of macroautophagy or microautophagy was occurring. The destruction of the proteins occurred in two distinct steps: first, the proteins noncovalently associated with lysosomes, and, second, they were translocated into the interior of the lysosome in an adenosine triphosphate (ATP)-dependent fashion.¹¹ Because the binding was saturable and competitive, specific binding of the protein to the lysosomal membrane accounted for the initial association. When ATP was depleted, the proteins bound to the outside of the lysosome, but were not translocated.¹² Inhibitors of lysosomal proteases did not affect association of the lysosomes or translocation, but rather led to accumulation of the proteins inside the lysosomes.

The name CMA arose from the discovery that the lysosomes from starved conditions had much greater amounts of heat shock cognate protein 70 (hsc70), a constitutively expressed member of the heat shock protein 70 chaperone family.¹³ Chaperones play key roles in folding, unfolding, and protein assembly by binding proteins of interest and recruiting co-chaperones. Hsc70 is a critical chaperone for a wide variety of different processes including clatherin-mediated endocytosis and certain types of microautophagy, and knock-out of hsc70 is embryonic lethal.¹⁴ Special isolation procedures targeting hsc70-containing lysosomes yielded high activity of specific protein uptake and destruction, whereas fractions lacking hsc70 were inactive.¹⁵ HSC70 is transported into active lysosomes, but it resists proteolysis by lysosomal proteases at low pH levels.^{16,17} Hsc70 specifically binds to CMA cargo proteins and the binding site differs in hsc70 from those used for other functions.

Proteins bound to hsc70 are unfolded and a complex of proteins, homologous to protein import complexes in mitochondrial and smooth endoplasmic reticulum membranes, is formed (Fig. 1).¹⁸ Hsc70 in this complex binds to the lysosome-associated membrane protein 2A (LAMP-2A) on the lysosomal membrane, which acts as the receptor, allowing import into the lysosome.^{19,20} Hsc70 increases inside the lysosome with increased CMA activity, but the internal hsc70 isoforms differ from those in the cytosol.^{2,16} This internal hsc70 is required for translocation, but not binding. In a lysosomal preparation, the total abundance of hsc70 identified with an antibody that detects all isoforms predicts CMA activity. Starvation can increase this abundance 4-fold in isolated liver lysosomes.¹⁷ Total cellular hsc70 does not change with CMA activity.

LAMP-2A abundance

In response to stimuli that increase autophagy in liver cells, LAMP-2A abundance increases, especially in the lysosomal membrane.²⁰ Importantly, modulation of lysosomal LAMP-2A levels in the lysosomal membrane either experimentally or through physiologic mechanisms modulate CMA activity, suggesting that lysosomal membrane LAMP-2A abundance is a rate-limiting step for the activity of the pathway.^{21,22} LAMP-2A is one of three protein products formed by alternative splicing of the LAMP-2 gene, which affects only the cytosolic portion of the protein, where it interacts with the hsc70–cargo protein complex.^{20,23} Fibroblasts isolated from mice with the entire LAMP-2 gene knocked-out show decreased macroautophagy as well as CMA.²⁴ When the hsc70–CMA cargo binds LAMP-2A, the monomers of LAMP-2A assemble into large molecular weight multimers of LAMP-2A into a high molecular weight complex (Fig. 1).^{25,26} This multimeric complex is required for translocation and is believed to be the transmembrane channel for translocation of cargo into the lysosomal lumen. After translocation the multimer disassembles, a process that also requires hsc70, before the monomers can bind another hsc70–cargo protein complex to repeat the cycle.

As the rate-limiting step in CMA, it is critical to understand how lysosomal membrane LAMP-2A (and thus CMA activity) can be regulated.² Lysosomal monomeric LAMP-2A is influenced by total cellular levels and by delivery to the lysosomal compartment, although these may be unreliable predictors of CMA activity. Although lysosomal LAMP-2A can be regulated in part by gene expression, CMA activity most often changes without measurable changes in LAMP-2A messenger RNA.²⁷ In cultured kidney cells and in whole kidney, total cellular LAMP-2A does not predict levels in isolated lysosomes during acute changes in CMA.^{28,29} However, after more than 24 hours of growth factor treatment in cell culture or after more than 3 days of diabetes, total cellular levels track lysosomal levels.^{28,29}

Lysosomal levels of monomeric LAMP-2A are acutely regulated to a much greater extent by proteolysis.^{26,27} Two proteases, a metalloprotease that cleaves the protein inside the lysosomal membrane and cathepsin A, which cleaves the intralysosomal portion of the protein, regulate LAMP-2A levels. The activity of both proteases destabilizes LAMP-2A, leading to its degradation and down-regulation of CMA activity. One mechanism for regulating LAMP-2A in starvation is controlling degradation by its regulatory protease, cathepsin A.³⁰ Lipid composition of the lysosomal membrane also alters the stability of LAMP-2A by promoting its movement to the specific lipid microdomains that are rich in

cathepsin A and metalloproteases.³¹ Regulation of the multimeric form of LAMP-2A by modulating its stability in association with lipid microdomains also has been described.²

CMA Cargo Proteins

CMA cargo proteins are defined by the hsc70 binding site, which initially was identified as a five-amino acid sequence similar to Lys-Phe-Glu-Arg-Gln (KFERQ).³ Polyclonal antibodies raised against KFERQ can prevent uptake of CMA cargo proteins into isolated lysosomes. However, other sequences (collectively called *KFERQ motifs*) are recognized as well as KFERQ. These contain a glutamine residue (Q) preceded or followed by 4 residues in any order: (1) positively charged (K or R), (2) negatively charged (D or E), (3) a bulky hydrophobic residue (I, L, V, or F), and (4) an additional positively charged or bulky hydrophobic residue.¹⁰ In some cases, an asparagine residue may substitute for the glutamine, but asparagine will not work with all sequences. The sequence must be on the exterior of the folded protein, accessible to hsc70. Proteins that are cleaved or unfolded may reveal KFERQ motifs on the interior of the protein. Protein modification near glutamine residues can create new motifs: acetylation can add a bulky hydrophobic residue, whereas phosphorylation or oxidation can add negative charges.² Only soluble cytosolic proteins that can be unfolded by hsc70 are believed to be substrates: transmembrane proteins and protein aggregates are resistant to CMA. Adding multiple KFERQ motifs does not appear to enhance the efficiency of CMA, but may allow for different forms of regulation upon unfolding or cleavage that shows KFERQ sequences. Multiple proven and predicted KFERQ-containing proteins play roles in renal diseases (Table 1).

Estimating CMA Activity

Traditionally, the gold standard for estimating the activity of CMA of tissue has been uptake assays testing the ability of isolated lysosomes to bind, translocate, and destroy CMA cargo proteins.³² The experiments are simple in concept: preparations of fresh lysosomes of equal purity between two conditions are compared in their ability to destroy radiolabeled or fluorescent-labeled substrate protein with KFERQ sequences. Because the individual proteins regulating CMA also may be studied in isolated lysosomes, the mechanism of the increase or decrease in CMA activity can be elucidated. This technique has been the basis of most advances in the field, however, the limitations of the techniques should not be underestimated. Because of the need for adequate starting material, uptake assays require a suitable quantity of homogenous tissue. The purity of the lysosomal fraction and the integrity of the lysosomal membrane are essential. Osmotic shock, such as that experienced after sucrose gradient isolation secondary to uptake of the sucrose into the lysosome, damages the lysosomes and metrizamide-based gradients are required. In addition, speed of isolation, temperature variations, and the source of metrizamide used in density centrifugation all effect CMA activity. The difficulty in obtaining a sufficient amount of isolated glomeruli or tubule segments means that all studies in the kidney have been performed in lysosomes isolated from renal cortex.²⁹

To avoid the difficulties of lysosomal uptake assays, expression vectors encoding fluorescent CMA cargo proteins have been developed.³³ These contain a KFERQ sequence and use a pH-sensitive fluorescence so that translocation into the acidic interior of

lysosomes as well as destruction can be monitored by confocal microscopy. The reporting of the intralysosomal position of the vector is crucial to exclude degradation outside lysosomes or in intralysosomal vesicles (ie, macroautophagy or microautophagy). This technique has not yet been applied to the kidney, but has great promise for studies in the many cell types of the kidney besides proximal tubule.

Less stringent assays also can be used to estimate relative CMA activity between different experimental conditions.³² Because LAMP-2A in the lysosomal membrane is the rate-limiting step for CMA, lysosomal LAMP-2A protein by Western blotting comparing different conditions can be used as a surrogate for the uptake assay to estimate relative CMA activity. Lysosomal hsc70 also correlates well with CMA activity. The use of both measurements adds confidence to the result and these measurements are performed easily in renal cortical or cultured proximal tubule lysosomes.^{28,29} The advantage of these assays is that protease inhibitors can be used throughout the purification to block autodigestion by the lysosomal enzymes. As discussed earlier, total cellular LAMP-2A is less useful, although we have found that in kidney cortex total cellular LAMP-2A correlates with lysosomal levels after chronic treatment.^{28,29}

For histologic studies, it has been noted that activation of CMA is associated with the mobilization of hsc70/LAMP-2A-enriched lysosomes to the peri-nuclear region of the cells.³² By using immunofluorescence or immunogold techniques the distance of the antibody staining to the nucleus can be measured. Co-localization of hsc70 and LAMP-2A also can be measured as another indication.

Lysosomal proteolysis measurements can be performed by studying the half-life of radiolabeled proteins with the use of proteolytic inhibitors to specify CMA or macroautophagy.^{28,32,34} Methylamine works well as the primary lysosome inhibitor and 3-methyladenine inhibits macroautophagy while leaving CMA unaffected. CMA is estimated as the proteolysis that resists both proteasome inhibitors and 3-methyladenine, but is sensitive to methylamine. The method may be used for cell culture for primary cultures or nontransformed renal tubular cell lines, but SV40-transformed renal tubular cell lines often undergo apoptosis in the presence of proteasome or lysosome inhibitors (personal unpublished observation). Note that the proteolytic activity of microautophagy is not addressed by these studies, but the specificity of the changes for CMA can be verified by examining the half-life of a specific KFERQ-containing protein, for example, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) in the presence of the inhibitors.^{28,32} Changes in the protein abundance by Western blotting of specific KFERQ proteins may suggest changes in activity, but also are subject to proteolysis by other systems and regulation by synthesis. For in vivo studies, we have adapted the technique of measuring total proteolysis for use in suspensions of isolated proximal tubule cells,^{29,35} but the study of the half-life of specific KFERQ proteins is not possible with this technique.

REGULATION OF CMA ACTIVITY

The rate of lysosomal proteolysis by CMA can be regulated by changes in the following: (1) the number of lysosomes active for CMA, (2) the rate of protein import into individual

lysosomes active for CMA, or (3) degradation within the lysosome (as regulated by lysosomal pH or enzyme content).³⁶ The lysosomal endopeptidases (cathepsins) require an acidic pH maintained by the vacuolar H⁺ ATPase.³⁷ Proteolysis in lysosomes can be inhibited by weak bases, such as ammonia, that increase lysosomal pH, or by inhibiting the vacuolar H⁺ ATPase with bafilomycin A1.^{38,39} Hsc70 is stable in CMA-active lysosomes at the normal low lysosomal pH level, but becomes unstable when the lysosomal pH increases.²⁷ In the kidney, ammonia may have a significant effect on CMA because 2 mmol/L NH₄Cl causes decreased proteolysis in primary rabbit proximal tubule cell culture and renal epithelial cell lines,^{40–42} while ammonia concentrations in the proximal tubule during metabolic acidosis or hypokalemia can reach 5 mmol/L.⁴³ In NRK-52E cultured renal tubular cells, ammonia decreased the lysosomal membrane abundance of LAMP-2A and increased the half-life of the KFERQ protein GAPDH.²⁸ Thus, high levels of ammonia in metabolic acidosis and hypokalemia may reduce CMA in the kidney.

Starvation has been used as a mechanism to investigate the signaling regulating CMA.³ In cell culture, ketone bodies provide a signal to activate CMA, suggesting that the timing of CMA activation with ketosis during starvation is not an accident.⁴⁴ Decreased glycolysis appears to be a signal for CMA activation because chemical inhibitors of glucose-6 phosphate dehydrogenase, a key glycolytic enzyme, activate CMA.⁴⁵ It is controversial if during starvation, loss of insulin signaling provides another stimulus for increasing CMA activity. In fibroblast cell culture, removal of serum or insulin increases CMA.⁴⁶ This led to speculation that type 1 phosphatidylinositol-3 (PI-3) kinase activity through the mammalian target of rapamycin (mTOR) would inhibit CMA in the same way that it does macroautophagy.^{34,47,48} However, in an embryonic fibroblast cell line studied under conditions of serum withdrawal, neither the PI-3 kinase inhibitor wortmannin, nor the mTOR inhibitor rapamycin had any effect on CMA.⁴⁵ Similarly, CMA is not affected by impairing the activity of class III PI-3 kinase with 3-methyladenine, a treatment that reduces macroautophagic activity.⁴⁵

In renal proximal tubular cells, growth factors in vitro and diabetes in vivo specifically suppress lysosomal proteolysis.^{49,50} Adding epidermal growth factor (EGF) to cultured NRK-52E renal tubular cells reduced lysosomal proteolysis, but neither rapamycin nor 3-methyladenine had any effect on lysosomal proteolysis in this system.³⁴ Macroautophagic activity is detectable in these cells, but, similar to the proximal tubule in vivo, occurs at a very low level,⁴ so the vast majority of the lysosomal proteolysis in these cells is caused by CMA. Lysosomal LAMP-2A is abundant in cultured proximal tubule cells and KFERQ-containing proteins are expressed at low levels.²⁸ The changes in proteolysis are specific to CMA because the lysosomal abundance of LAMP-2A decreases and the half-life of KFERQ proteins GAPDH and the paired box transcription factor, pax2, increases.

Similar to insulin, EGF signals through its receptor and activates a number of pathways, of which the Ras-activated signaling molecules, mitogen-activated protein kinase, and PI-3 kinase are critical for growth (Fig. 2).⁵¹ Expression of a dominant-negative Ras in NRK-52E cells prevented EGF from reducing proteolysis.⁴⁸ Inhibitors of type 1 PI-3 kinase, but not of mitogen-activated protein kinase prevented suppression of lysosomal proteolysis. The type 1 PI-3 kinase inhibitor LY294002, or expression of a dominant-negative p85 subunit of type 1

PI-3 kinase prevented EGF from decreasing proteolysis or increasing accumulation of the KFERQ-containing proteins, whereas rapamycin, which prevents type 1 PI-3 kinase from reducing macroautophagy, did not. Thus, unlike serum deprivation in fibroblasts, PI-3 kinase appears to regulate CMA in renal tubular cells.

Because glycolysis regulates CMA and proximal tubule cells have low levels of basal glycolysis,^{45,52} we examined signaling downstream of class 1 PI-3 kinase through Akt/protein kinase B and subgroup O1 of the forkhead-related transcription factors (FoxO1). Akt directly up-regulates cell glucose uptake and increases glycolysis, in part, by suppressing FoxO1.^{53–55} Akt phosphorylation of FoxO transcription factors lead to their phosphorylation and nuclear exclusion and proteasomal degradation. Expressing a dominant-negative Akt prevented EGF from reducing lysosomal proteolysis or increasing the protein levels of KFERQ proteins pax2 and GAPDH, while using a constructively active Akt without EGF-reduced proteolysis and increased pax2 and GAPDH.³⁴ Expressing a constitutively active FoxO1 had the opposite effect: it prevented EGF from reducing lysosomal proteolysis and decreased EGF-induced expression of GAPDH and pax2 (personal unpublished data). FoxO3a is involved in up-regulation of macroautophagy and is regulated by Akt in the same fashion.⁵⁶ Measuring CMA activity as perinuclear localization of LAMP-2A, siRNA against FoxO1, but not siRNA against FoxO3a, reduced CMA activity. FoxO1 also can regulate macroautophagy,⁵⁷ but we found no evidence of altered macroautophagy with siRNA against FoxO1. In renal tubular cells, Akt/FoxO1 regulates CMA.

Despite the evidence that CMA may be a response to decreased glycolysis, the effect of CMA is dependent on the particular KFERQ substrate proteins expressed in a cell. Different isoforms of glycolytic enzymes in different cells means that many are resistant to degradation by CMA.⁵⁸ In fact, the relationship between glycolysis and CMA is reversed in many cancer cells.⁵⁹ The M2 isoform of pyruvate kinase (M2PK), a glycolytic enzyme widely produced in cancer, is inactivated by acetylation, but still competes for substrate with the active form of the enzyme. Thus, acetylation of M2PK creates a competitive inhibitor slowing glycolysis. However, the acetylation of M2PK creates a new KFERQ motif, so that CMA destroys the inactive inhibitory form of the enzyme. In this way, increased CMA activity actually increases glycolysis in cancer cells.

Dietary lipids are another regulator of CMA.⁶⁰ Lysosomal membrane LAMP-2A associates with cholesterol and sphingolipid enriched lipid microdomains and undergo degradation by proteases.³¹ LAMP-2A moves to these high-cholesterol regions when CMA activity is low, but it remains outside the domains when CMA is active. When young mice received high-cholesterol or high vegetable oil and lard diets for 3 weeks, CMA activity decreased in the liver of these animals.⁶¹ Lysosomal LAMP-2A was reduced in the liver of these animals as a consequence of accelerated degradation in lysosomes. Because the liver has a unique role in dietary fat metabolism, it is not clear whether this maneuver would alter CMA in other organs. However, the lipid profile of lysosomes from the fat-fed mice was similar to that seen in lysosomes from aging animals, in which reduced LAMP-2A also arose from increased instability of this receptor at the lysosomal membrane.² Because lipid changes in the kidney are hallmarks of kidney dysfunction in diabetes and aging,⁶² these mechanisms may influence renal disease in these settings.

Another major factor regulating CMA activity is the activity of other proteolytic pathways. When cultured cells have macroautophagy induced by mTOR inhibition or by other means, there is an increase in other proteolytic pathways, including CMA.⁶³ When macroautophagy is inhibited by siRNA against components of the macroautophagic pathway, there is a compensatory up-regulation of CMA. This relationship has been confirmed in vivo using a Huntington disease mouse model.⁶⁴ In this model, when macroautophagy was inhibited there was both transcriptional up-regulation and down-regulation of degradation of LAMP-2A to increase the activity of CMA. CMA also increases when the ubiquitin-proteasome system is inhibited.³³ Substrate availability has been suggested as important in regulating cross-talk between proteolytic systems, but no mechanism has been put forward to explain how decreased macroautophagy increases CMA. CMA activity also regulates macroautophagy: increasing or decreasing CMA activity by manipulating LAMP-2A levels causes the inverse change in macroautophagic flux.⁶³ There is a possible mechanism for this regulation, macroautophagic cargo recruits p62/sequestosome-1, which can stimulate macroautophagy.⁶⁵ In preliminary data, the same cross-talk occurs in renal tubular cells where the decrease in CMA in early diabetes is accompanied by an increase in macroautophagy (personal unpublished data).

Another situation in which an increase in CMA activity is associated with increased substrate is in oxidative stress.⁶⁶ As mentioned earlier, the KFERQ motif relies on one negatively charged amino acid residue, and oxidative modification of amino acids places new negative charges. Oxidation thus creates new substrates for CMA and allows lysosomal import of modified proteins that are not otherwise CMA substrates. Furthermore, there is increased capacity for degradation because increased stability of lysosomal LAMP-2A drives its increased abundance. In addition, in severe oxidative stress, increases in LAMP-2A messenger RNA occur, further up-regulating CMA.⁶⁶ The up-regulation of CMA by oxidative stress is an important survival response⁶⁷ and accumulation of oxidized proteins occurs when CMA is down-regulated in a variety of settings including aging and diabetes.⁶⁸ The response to oxidative stress may be part of an overall response to cell stress because endoplasmic reticulum stress also may increase CMA activity.²

Finally, a recent report showed that signaling through the all-trans retinoic acid receptor (RAR) α , inhibits CMA.⁶⁹ Knocking down or inhibiting RAR α with small-molecule inhibitors increases lysosomal destruction of a fluorescent KFERQ-containing CMA reporter gene and increases lysosomal LAMP-2A, in part through a transcriptional mechanism. Because retinoic acids are important modulators of differentiation and proliferation, RAR α provides another global control of the CMA system.⁷⁰ Loss of RAR α occurs in many cancer cells and it will be of interest to see if this correlates with activation of CMA in cancer cells.

SIGNIFICANCE OF CMA

Starvation

CMA preferentially targets longer-lived proteins for destruction.³⁶ In starvation, macroautophagy in the liver is activated in the first 6 hours followed by activation of chaperone-mediated autophagy by 12 hours, becoming maximal at 24 hours, and continuing

for up to 3 days.⁷ The selectivity of CMA is based on the KFERQ motif, so there is no mechanism, as seen with the proteasome, such that only a few proteins may be targeted at a time. Because of the large number of proteins with the KFERQ motif, destruction by CMA tends to cause only a 50% to 150% change in targeted protein half-lives.^{28,71} For example, the transcription factor c-fos is destroyed by both the proteasome and CMA.¹³ C-fos has a rapid increase in its abundance after a mitogenic stimulus, which is terminated by proteasomal degradation over approximately 1 hour. However, the steady-state level of c-fos after the acute increase is regulated by CMA such that the half-life of c-fos changes from approximately 8 to 13 hours. Thus, CMA provides chronic regulation of many specific proteins, but does not appear to be involved in the minute-to-minute responses of particular proteins.² This global regulation of cell protein metabolism has focused research on longer-term processes, such as starvation, oxidative stress, cell growth and neoplasia, aging, and chronic disease.

The percentage of cellular proteins that is potential cargo for CMA is difficult to answer precisely because gene expression patterns differ between and within tissues.⁹ The estimate, based on antibody binding to KFERQ motifs of partially denatured protein, is that up to 30% of cellular protein may be substrates. It is not clear if all those motifs are available for binding without denaturation or cleavage of the protein. Interestingly, liver and renal proximal tubule have the highest percentage of proteins (~30%) with KFERQ motifs and high basal levels of CMA activity.⁹ However, in animal models, protein or calorie starvation further increases CMA in the kidney.³ During the first week of starvation, kidney and liver preferentially lose protein, whereas tissues with lower levels of KFERQ proteins, such as muscle, are spared.⁷² The destruction of proteins by CMA during starvation does seem to serve cellular energy requirements because starved cells have lower ATP levels compared with those with intact CMA.²¹

CMA in Renal Tubular Hypertrophy

Why is CMA important for understanding renal tubular hypertrophy? Renal tubular cells accumulate the protein needed for growth in part by activation of classic growth factor-activated pathways of protein synthesis, but also through suppression of lysosomal pathways of protein degradation.^{43,73,74} In hypertrophy caused by metabolic acidosis, there is no net increase in protein synthesis, but only a decrease in protein degradation.^{5,75} Basal levels of macroautophagy are low in proximal tubule, therefore suppression of lysosomal proteolysis primarily leads to reductions in CMA.³⁴ In models of acute type 1 diabetic renal hypertrophy, a burst of protein synthesis occurs over the first 7 days, but then synthesis returns to baseline levels.^{29,76} However, proteolysis is decreased by approximately 40% by day 3 and continues to be suppressed for approximately 6 weeks. In models of metabolic acidosis, protein synthesis is unchanged: there is only a reduction in proteolysis.⁷⁵ As discussed earlier, both ammonia and growth factors believed active in diabetic renal hypertrophy (EGF, hepatocyte growth factor, and insulin like growth factor 1) specifically suppress lysosomal proteolysis.⁴⁹ In most cell types, growth factors suppress lysosomal proteolysis through macroautophagy,^{77,78} but there is a low level of macroautophagy in nontransformed proximal tubular cells in vivo.⁷⁹ In NRK-52E cells, down-regulation of macroautophagy with mTOR and type III PI-3 kinase inhibitors does not change basal levels

of lysosomal proteolysis,³⁴ suggesting that CMA is the dominant lysosomal proteolytic process.

In tissue culture, any lysosomal inhibitor is sufficient to induce hypertrophy in cultured renal tubular cells.^{40,42} Many isoforms of enzymes abundant in renal hypertrophy contain a KFERQ CMA-targeting sequence, including phosphofructokinase, GAPDH, pyruvate kinase, choline kinase, and phosphatidylcholine transferase (Table 1).^{80,81} Notably, the KFERQ motif in these proteins is conserved across vertebrate species, is accessible to binding by heat shock protein 70, and many of these proteins are degraded in response to serum removal. Many transcription factors that regulate renal tubular cell growth are proven to be destroyed by CMA including pax2, c-fos, and hypoxia-inducible factor-1 α , and others, such as Pax8, have KFERQ sequences.^{5,82} The addition of ammonia to the media of cultured renal tubule cells is sufficient to up-regulate pax2 protein levels.²⁸ In cultured renal tubule cells, ammonia or EGF decrease CMA as assessed by decreased lysosomal LAMP-2A levels and increase the half-life of GAPDH. Thus, CMA is reduced in cell culture models of renal tubular hypertrophy.

CMA also was decreased in the renal cortex in an experimental model of early type 1 diabetes.²⁹ In the renal cortex from rats made diabetic with streptozotocin injection, lysosomal proteolysis decreased at 7 days and LAMP-2A from isolated renal cortical lysosomes was also decreased.²⁹ Pax2 was not detectable in lysates or nuclear extracts of untreated rat renal cortex, but could be detected in isolated renal cortical lysosomes. This suggests that CMA helps regulate the abundance of pax2 in normal conditions. In the 7-day diabetic rats, Pax2 was measurable in lysates and abundant in nuclear extracts, but the amount of pax2 protein in the lysosome did not change, suggesting that lysosomal uptake decreased with diabetes. The increased nuclear abundance resulted in increased pax2 DNA binding activity in nuclear extracts from diabetic rats. Decreased CMA activity seen in diabetes contributes to the overall decrease in proteolysis and accumulation of proteins important for renal tubular cell growth.⁸³

Cancer

Despite CMA's role to suppress glycolysis and growth in liver and proximal tubule cells, increased CMA activity plays a major role in cancer.⁵⁸ As discussed earlier, CMA activity is up-regulated in many cancer cell lines, and levels of LAMP-2A are also higher in human tumor samples than in the normal tissue where the tumor arose.⁵⁹ When CMA in tumor cell lines was reduced by genetic manipulation of LAMP-2A, cancer cell proliferation decreased. When a human lung cancer cell line with genetically reduced CMA was implanted in mice, tumor growth and generation of metastasis was impaired. The expression of isoforms of growth-related proteins, especially glycolytic proteins, that resist degradation by CMA (or in the case of M2PK are activated by CMA), appear to be responsible for this effect. Furthermore, CMA provides a survival advantage, perhaps owing to oxidative and endoplasmic reticulum stress in these tumor cells.⁸⁴

Although CMA activity aids cancer progression, CMA activity should have an anti-oncogenic effect in renal tubular cells. The most common renal cell carcinoma, clear cell, arises because of mutations to the von Hippel-Lindau gene product.⁸⁵ The von Hippel-

Lindau gene product targets potential oncogenes, the hypoxia-inducible factors, for proteasomal destruction. The KFERQ motif targeting hypoxia inducible factor 1 α targets the protein for CMA, providing a secondary means to suppress this protein.⁸² In addition, CMA targets destruction of epidermal growth factor–receptor pathway substrate 8, which contributes to the promotion of renal cell carcinoma, among other malignancies.⁸⁶

Oxidation in Aging and Diabetes

Oxidative stress is one of the major activators of CMA, and if CMA is impaired during oxidative stress then cell viability suffers.²¹ In aging, there is accumulation of oxidized, otherwise denatured, or glycosylated proteins and aggregates of oxidized proteins that form substrates for macroautophagy.⁸⁷ Dietary calorie restriction helps prevent the accumulation of these substances and the activation of CMA and macroautophagy by calorie restriction is one proposed mechanism for this improvement.⁸⁸ In chronic kidney disease, protein nitrosylation becomes a significant source of aggregates.⁸⁹ There are profound age-related changes in the lysosomes used by macroautophagy with accumulation of lipofuscin, suggesting incomplete degradation of proteins.⁸⁷ The age-related loss of CMA results from loss of lysosomal LAMP-2A.⁹⁰ There is an increase in the number of lysosomes active for CMA in aging, but because of their lower LAMP-2A content, older lysosomes have decreased CMA activity.⁸⁷ It is believed that the lipid composition of the older lysosomes drives rapid degradation of LAMP-2A.⁹¹ The down-regulation of CMA is accompanied by an up-regulation of macroautophagy, likely driven in part by the increasing number of protein aggregates.²¹ Mono-ubiquitination of the proteins leads to p62/sequestosome 1 labeling of the protein aggregates, which drives macroautophagy.⁶⁵ Because aging down-regulates macroautophagy in liver and kidney, the aggregates accumulate.^{92,93} The oxidized and advanced glycosylation end products in the aggregates act as a repository of oxidative stress, leading to additional protein and DNA damage. The end result is a compromise of cellular function and ultimately viability.²¹

This raises the question about the significance of the decrease in CMA. If CMA were restored, could tissue damage be prevented? To answer this question, Zhang and Cuervo⁹³ created a transgenic mouse model in which an exogenous copy of LAMP-2A was expressed in liver with an inducible albumin promoter. The inducible promoter was activated just enough to compensate for the decrease in the endogenous LAMP-2A with aging. Expression of this transgene successfully restored lysosomal LAMP-2A and CMA activity in the older mice to the same level as the younger mice. Activation of this transgene reduced levels of oxidized proteins as assessed by carbonyl proteins and p62-labeled aggregated proteins in liver. Interestingly, there was evidence of improved hepatic function: recovery time from a hepatically metabolized anesthetic improved to the level seen in a young mouse. This result suggests that the decrease in CMA with age is functionally significant in terms of oxidized protein accumulation and loss of organ function.

To examine if decreased CMA in early diabetes is associated with increased oxidation or accumulation of aggregates, we examined markers of autophagy and oxidative stress in rat renal cortex 21 days after streptozocin-induced diabetes (personal unpublished data). As expected, when CMA was decreased, oxidized proteins increased and macroautophagy had a

compensatory increase. Unlike aging, in early diabetic renal hypertrophy there was no increase in p62/sequestosome 1-labeled aggregates.⁹² To evaluate the role of decreased CMA, we administered high- and low-protein diets. The high-protein diet would suppress CMA by increasing ammonia production, and the low-protein diet would stimulate CMA.^{9,83} Diabetic rats on the high-protein diets had slightly lower blood sugar levels, but significantly lower levels of LAMP-2A, higher levels of oxidized protein, and higher levels of macroautophagy when compared with rats on the lower-protein diet. Again, there was no change in p62-labeled aggregates. Suppressing CMA was associated with increased renal cortical oxidation and macroautophagy, but the increase in macroautophagy was sufficient to prevent a statistically significant increase in p62. It is possible that as diabetes continues, similar to aging, the high levels of oxidized proteins will cause aggregate formation and enhance tissue damage (Fig. 3). Furthermore, reduced CMA creates an additional mechanism to explain the effects of dietary protein and calories on risk of progression of renal disease.⁸⁹

Neurodegeneration and Proteotoxicity

Because CMA has the ability to destroy soluble proteins before they aggregate, there has been a flurry of interest in the concept of CMA protecting against proteotoxicity in age-related, neurodegenerative disease.⁶⁴ The common theme in these diseases is that CMA is able to destroy a component of the pathogenic proteins that form inclusions inside or outside neurons. KFERQ motifs targeting CMA are present in many Parkinson disease-related proteins (α -synuclein, parkin, neuronal ubiquitin C-terminal hydrolase-L1, pink-1, and parkinsonian protein 7), Alzheimer disease-related proteins (amyloid precursor protein and Tau), and in huntingtin, the protein mutated in Huntington disease. A large series of elegant studies have identified that decreases in CMA with aging are one contributing factor to the failure to degrade these proteins and in the formation of protein aggregates. Thus, the loss of CMA activity with aging has major implications for the formation of protein aggregates and overall pathogenesis in these neurodegenerative diseases.

There are no known renal diseases with CMA-sensitive, age-induced inclusions. The one described example of renal proteotoxicity occurs as a result of increased rather than decreased CMA. Hyaline droplet nephropathy (also called α_2 -microglobulin nephropathy), which occurs after exposure to a variety of aliphatic, alicyclic, or aromatic compounds (ie, hydrocarbons), is associated with acute kidney injury and the possibility of renal tubular carcinogenesis during recovery.⁹⁴ The hyaline droplets that form inside tubular cells are actually lysosomes with a high concentration of α_2 -microglobulin. Exposure to hydrocarbons induces increased lysosomal LAMP-2A, presumably by inducing oxidative stress.⁹⁵ The up-regulation of CMA induces the lysosomal uptake of α_2 -microglobulin that has interacted with the hydrocarbons to make it a CMA substrate. α_2 -Microglobulin is degraded poorly by lysosomal enzymes, so it accumulates in the lysosomes, becoming the major protein of the lysosome. The resulting lack of lysosomal function (including, ironically, a decrease in CMA activity) in the presence of oxidative stress induces proteotoxicity and acute tubular necrosis. The mechanism of carcinogenesis presumably is related to abnormal regrowth, but it is not clear if decreased CMA activity contributes.

CMA IN OTHER KIDNEY DISEASES

Renal dysfunction may be seen in a variety of different lysosomal storage diseases, but the role of CMA in the pathology of these diseases remains unclear.⁹⁶ Certainly there is the potential for decreased CMA with profound lysosomal dysfunction. In mucopolidosis type IV, for example, cells show decreased lysosomal LAMP-2A and this decrease may contribute to tissue oxidation.⁹⁷ However, one disease, galactosialidosis, is of particular interest because one causative gene is the LAMP-2A protease, cathepsin A.⁹⁸ Of the different genes mutated in galactosialidosis, only some, but not all, patients with disease-caused cathepsin A mutations have a severe infantile form associated with small kidneys and death from renal failure, hypertension, and cardiovascular disease in the first year of life.^{98,99} Cathepsin A knockout mice have the same phenotype as the severe infantile form: death from renal failure at an early age.⁹⁸ Interestingly, these knockout mice lacking the regulatory protease of LAMP-2A have constitutively active CMA from a decreased ability to down-regulate LAMP-2A.³⁰ Because CMA target protein pax2 is critical for branching and nephron number during renal development,¹⁰⁰ further study is needed to determine if up-regulation of CMA during renal development contributes to the kidney disease.

CONCLUSIONS

CMA, a selective form of degradation of long-lived proteins, is important in the renal proximal tubule, which has high basal levels of CMA activity. In the proximal tubule, CMA clearly impacts renal hypertrophy, diabetes, aging, and storage diseases, but the clinical usefulness of approaches targeting CMA in these diseases remains to be determined. Restoration of CMA function may improve alterations with aging and may provide a link between diet and aging, as well as diet and chronic kidney disease. CMA has not been examined in detail in the glomerulus, distal nephron, development of renal cancer, or morphogenesis and development of the kidney. It is of interest that KFERQ motifs appear in proteins having important roles in renal disease including the pax2 and pax8,²⁸ NPHS2 gene product, podocin,⁴ and hypoxia-inducible factor 1 α .⁸² With new tools for the measurement of CMA activity and new ways to regulate CMA activity in vivo, the question of the role of CMA in renal diseases finally seems answerable.

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Figure 1.

A diagram of some events in CMA. (1) Hsc70 binds the CMA recognition sequence (KFERQ) on a cytosolic protein. (2) Co-chaperones bind hsc70, denaturing the protein. (3) Hsc70/co-chaperones/protein bind LAMP-2A on the lysosomal membrane. (4) LAMP-2A forms a multimeric complex to form a pore while co-chaperones and both luminal and cytoplasmic hsc70 translocate the protein across the membrane. (5) The protein is degraded and the complex disassembles completely, often in lipid microdomains where cathepsin A is active. (6) LAMP-2A is either recycled for further CMA or degraded first by cathepsin A and subsequently lysosomal proteases to down-regulate CMA.

Figure 2.

EGF-related signaling pathways in renal epithelial cells regulating CMA. Pathways promoting synthesis (green) and promoting proteolysis (red) interact. After EGF activates Rous Sarcoma protein (Ras), the mitogen-associated protein kinase (MAPK), regulates protein synthesis only. Type 1 PI-3 kinase increases protein synthesis and down-regulates macroautophagy through Akt/protein kinase B and mTOR. Akt also inhibits FoxOs to down-regulate macroautophagy through FoxO1 and FoxO3a and CMA through FoxO1.

Figure 3.

Inhibition of CMA in proximal tubule during diabetes increases macroautophagy and may promote tissue damage. Diabetes creates oxygen free radicals, which can damage proteins by oxidation. When CMA is decreased in the proximal tubule during diabetes, destruction of cytosolic oxidized proteins decreases. There is increased protein aggregation of these damaged proteins, leading to p62/sequestosome 1 binding to the aggregates. The p62 stimulates macroautophagy, removing most of the oxygen free radicals, but some may escape from the aggregates to provide continued oxidative stress and tissue damage.

Table 1

Selected CMA Substrates Relevant to Renal Disease

Proven Substrates	Predicted From Sequence
Aldolase B	Choline kinase
Annexin I	Phosphorylcholine transferase
Annexin II	Phosphofructokinase
Annexin IV	Pax-8
Annexin VI	Park-7 (DJ-1)
Aspartate aminotransferase	Podocin
c-fos	UCH-L1
EPS8	
Galectin-3	
GAPDH	
Glutathione transferase	
HIF-1 α	
I κ B	
LRRK-2	
MARKS	
α 2-microglobulin	
Pax-2	
Pyruvate kinase	
RCAN1	

c-fos, oncogenic factor; EPS8, epidermal growth factor receptor pathway substrate 8; HIF-1 α , hypoxia inducible factor-1 α ; I κ B, inhibitor of the nuclear factor- κ B; LRRK-2, leucine-rich repeat kinase 2; MARKS, microtubule affinity regulating kinase substrate; Pax, paired box-related transcription factor; RCAN1, regulator of calcineurin 1; UCH-L1, neuronal ubiquitin C-terminal hydrolase-L1.