The Role of Ubiquitin-Specific Protease 14 in Glomerular Injury

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Table of Contents

Abstract

Protein misfolding in the endoplasmic reticulum (ER) of glomerular epithelial cells (GEC)/podocytes is a feature of certain types of glomerulonephritis. In experimental membranous nephropathy, complement C5b-9 induces sublethal GEC injury and proteinuria. In parallel, C5b-9 activates pathways to restrict injury. Nephrin is an important component of the podocyte slit diaphragm, and mutations in nephrin lead to proteinuria and congenital nephrotic syndrome. Nephrin mutants are misfolded, engage ER chaperones, accumulate in the ER and further contribute to ER stress. We tested if enhancement of ubiquitin-proteasome system (UPS) function may confer protection against GEC injury and ER stress. Certain deubiquitinating enzymes, including ubiquitin-specific protease14 (Usp14), retard protein degradation by the proteasome. Thus, inhibition of Usp14 may potentially enhance degradation of misfolded proteins, and attenuate cell injury. In GECs, the reporter proteins, GFP^{u} (a misfolded protein) and CD3δ-YFP (an ER-associated degradation substrate) undergo time-dependent proteasomal degradation. Incubation of GEC with complement did not affect expression of CD3δ-YFP significantly, but accelerated degradation of GFP^{u}, and the Usp14-directed inhibitor, IU1, further accelerated this degradation. Conversely, overexpression of Usp14, but not a catalytically-inactive Usp14 mutant, reduced degradation of GFP^{u} and CD3δ-YFP. IU1 did not alter complement-induced cytotoxicity in GECs. In Human embryonic kidney 293T cells, IU1 did not enhance degradation of the human disease-associated nephrin missense mutants, I171N and S724C, whereas overexpression of Usp14 delayed their degradation. IU1 did not modify global ubiquitination of proteins. IU1 protected cells from cytotoxicity after ER-stress induction by intermediate doses of tunicamycin. In conclusion, Usp14 controls proteasomal degradation of some misfolded proteins. Inhibition of Usp14 appears to be useful in alleviating the proteotoxic effects of specific misfolded proteins. In addition, Usp14 inhibition is effective in reducing cytotoxicity in the context of global protein misfolding during certain types of cell injury.

Résumé

Le repliement anormal des protéines dans le réticulum endoplasmique des cellules glomérulaires épithéliales (CGE) /podocytes est une caractéristique de certains types de glomérulopathies. Dans le cas de la glomérulonéphrite membraneuse expérimentale, le complément C5b-9 induit une lésion sublétale des CGE ainsi que de la protéinurie. Parallèlement, C5b-9 active également des voies métaboliques qui limite les dommages cellulaires. La néphrine est une composante importante des fentes de filtration des podocytes. Des mutations de la néphrine entraînent de la protéinurie et le syndrome néphrotique congénital. Les mutations de la néphrine engendrent un mauvais pliage de la protéine, stimulent les chaperons du réticulum endoplasmique, s'accumulent dans et contribuent au stress du réticulum endoplasmique. Nous avons testé si un renforcement du système ubiquitine-protéasome permet une meilleure protection contre les lésions des CGE et du stress du réticulum endoplasmique. Certaines enzymes désubiquitinisantes, telles que l'Usp14 (ubiquitin-specific protease 14), retardent la dégradation des protéines par le protéasome. En conséquence, l'inhibition de l'Usp14 pourrait augmenté la dégradation des protéines repliées anormalement et atténuer les lésions cellulaires. Dans les CGE, les protéines rapporteuses GFP^u (une protéine repliée anormalement) et CD3δ (un substrat de dégradation associé au réticulum endoplasmique) subissent une dégradation mésurable avec le temps, par le protéasome. L'incubation des CGE en présence du complément n'a pas modifié l'expression de CD3δ de façon significative, mais nous avons noté une accélération de la dégradation de GFP^u. L'utilisation de l'inhibiteur d'Usp14, IU1, a augmenter davantage cette dégradation. Inversement, la surexpression de Usp14 a réduit la vitesse de dégradation de GFP^u et CD3δ, ce qui n'a pas été observé lors de la surexpression d'un mutant catalytiquement inactif de Usp14. IU1 n'a pas eu d'effet sur la cytotoxicité induite par le complément sur les CGE. Dans les cellules de rein embryonnaires humaines 293T, IU1 n'a pas accéléré la dégradation des mutants I171N et S724C de la néphrine (ces mutants faux-sens sont associés à des glomérolupathies chez l'humain), par contre, la surexpression de Usp14 a retardé leur dégradation. IU1 n'a pas modifié l'ubiquitination de l'ensemble des protéines. IU1 a eu un effet protecteur contre la cytotoxicité induite par des doses intermédiaires de tunicamycin, un inducteur de stress du réticulum endoplasmique.

On peut conclure que l'Usp14 contrôle la dégradation protéosomique de certaines protéines repliées anormalement. L'inhibition de l'Usp14 semble être utile en atténuant l'effet protéo-toxique de certaines protéines mal repliées. L'inhibition de l'Usp14 réduit également de façon efficace la cytotoxicité dans le contexte global du repliement erroné des protéines pour certains types de lésions cellulaires.

Table of Abbreviations

ATF	Activating transcription factor	**LDH**	Lactate dehydrogenase
BiP	Binding immunoglobulin protein	**MAC**	Membrane attack complex
CNX	Calnexin	**MN**	Membranous nephropathy
cPLA$_2$	Cytosolic phospholipase A$_2$	**NS**	Normal Serum
CRT	Calreticulin	**PERK**	Protein kinase R-like ER kinase
DUB	Deubiquitinating enzyme	**RPN11**	Regulatory proteasomal subunit 11
ER	Endoplasmic reticulum	**UBD**	Ubiquitin binding domain
ERAD	ER-associated degradation	**Ubl**	Ubiquitin-like domain
ERQC	ER quality control	**UBP**	Ubiquitin-specific processing proteases
GBM	Glomerular basement membrane	**Uch37**	Ubiquitin C-terminal hydrolase 37
GEC	Glomerular epithelial cells	**UPR**	Unfolded Protein Response
GFP	Green-fluorescent protein	**UPS**	Ubiquitin-Proteasome System
GRP	Glucose-related protein	**Usp14**	Ubiquitin-specific protease 14
GSD	Glomerular slit diaphragm	**Usp14CA**	Usp14C114A
HIS	Heat Inactivated Serum	**XBP1**	X-box binding protein 1
IRE1α	Inositol-requiring enzyme 1α	**YFP**	Yellow-fluorescent protein
IU1	Small-molecule inhibitor of Usp14		

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Chapter 1: Introduction

The Kidney and Podocytes

The kidneys are made up of about a million functional units, called nephrons. The nephron lies in two structurally different regions of the interior of the kidney: the superficial cortex and the deep medulla. The nephron is the primary unit of filtration of the kidney, and consists of a renal corpuscle and a tubule. The renal corpuscle consists of the glomerular tuft, where the blood enters to be filtered, and the Bowman's space, where the filtrate collects and goes to the tubules, for electrolyte balancing, concentration and collection, to form urine. This cell and protein-free urine is then expulsed from the body through the bladder and ureter [1].

The glomerular tuft, or glomerulus, is a network of capillaries formed by the branching of the afferent arteriole. The glomerulus is surrounded by the glomerular basement membrane (GBM) as a structural scaffold. Four cell types comprise the glomerulus: endothelial cells, mesangial cells, parietal epithelial cells of the Bowman's capsule and podocytes. Podocytes, or glomerular epithelial cells (GEC), are highly specialized and polarized cells that line outer part of the GBM, while endothelial and smooth muscle-like mesangial cells line the inside of the GBM. Podocytes have a complex cellular organization and consist of a cell body and foot processes. These podocyte foot processes wrap around the capillaries in an interdigitating pattern with adjacent podocytes, forming the filtration slits that comprise the glomerular slit diaphragm (GSD) [2]. The GSD covers the outer part of the GBM and is the major player in determining the selective permeability of the glomerular filtration barrier, normally only freely permeable to water and small solutes [3]. Therefore, it follows that podocyte injury, reflecting a "leaky" filtration barrier, is associated with albuminuria. As is the case with many highly differentiated cells, the podocyte has limited ability to undergo cell division in cases of loss of podocytes: a hallmark of progressive kidney disease. Preventing or rescuing podocytes from injury is therefore obvious target of research for treatment of various kidney diseases [2].

Complement

The complement system comprises of approximately 30 soluble and membrane-bound proteins and is an important part of the body's innate immune system, involved in, the destruction and removal of pathogens, apoptotic cells and immune complexes. Complement activation can occur via three pathways, which consist of: the classical, the alternative and mannose-binding lectin pathways. The three pathways intertwine at a vital complement component, C3. Overall, C3 cleavage by C3 convertase leads to the downstream generation of C5, which in turn leads to the assembly of the membrane attack complex (MAC) C5b-9, releasing anaphylotoxins C3a and C5a, and deposits opsonins C3b and C4b in the process [4]. C3a mediates local inflammation and C3b opsonizes pathogenic cells for destruction. MAC C5b-9 forms pores in the membrane and proceeds to injure or lyse cells [5].

Functions of complement include: opsonization, enhancement of phagocytosis, chemotaxis, cytolysis, solubilization of immune complexes and regulating the acquired immune response. This physiological response is crucial for the natural defense against pathogens, but an excessive activation of complement can be pathological and contribute to damage of host tissues. The kidneys are particularly susceptible to cellular and tissue damage by complement activation and complement-mediated injury is associated with many renal diseases. Glomerulonephritis (inflammation of the glomerulus) is characterized by glomerular deposits, which contain C3 fragments, and presents with heavy proteinuria and continuing decline in renal function. In many cases immune complexes form in the glomerular capillary wall, resulting in complement activation. Antibody-mediated cellular injury in glomerulonephritis typically occurs in three ways; i) antibodies react with glomerular antigens already found in the GBM or glomerular cell membranes, ii) circulating antigens get "planted" in the glomerular capillary wall and then bind with antibodies, or iii) via circulating antigen-antibody complex deposition. The most damaging antibody-antigen complex formation occurs on the GBM near the capillaries, resulting in C5a attracting the invasion of immune cells and components from the blood. This leads to C5b-9 activation on the podocytes and their foot processes, leading to podocyte injury, disruption of the filtration barrier and results in proteinuria [5].

Membranous Nephropathy

One of the most common forms of nephrotic syndrome in adults is membranous nephropathy (MN), accounting for about one fifth of all cases. Most cases are idiopathic, or secondary to infections, lupus erythematous, cancer and drug intoxication. One of the most prominent characteristics of membranous nephropathy is immune-complex deposition in the subepithelial space, causing podocyte injury and proteinuria. Eventually, a membrane-like thickening of the GBM occurs. The immune deposits consist of IgG, C5b-9 membrane attack complex and other antigens [6].

The pathogenesis of MN has been understood from studying the passive Heymann nephritis (PHN) model of MN in the rat, due to its clinical and pathological similarities to the human disease. In experimental MN, antibodies bind to GEC membrane antigens, activate complement, and leads to assembly of the C5b-9 membrane attack complex, which results in sublethal GEC injury and proteinuria. Upon sublethal C5b-9 attack, the podocyte activates several factors including, but not limited to, protein kinases, phospholipases, cyclooxygenases, transcription factors, growth factors, NADPH oxidase, stress pathways, proteinases etc., which impact metabolic pathways, the function and structure of the cytoskeletal and matrix and membrane components [7]. Additional effects of C5b-9 include loss of expression of nephrin, a crucial slit diaphragm protein [8] and reduction in F-actin-bound nephrin, further impacting slit diaphragm integrity and increasing adverse effects on podocytes [7].

During the process of sublytic injury and overall loss of membrane integrity in complement-mediated glomerular disease, cellular defense signaling pathways are activated. In cultured rat GEC, C5b-9 activation leads to the activation of protein kinase C, phospholipase C, increased intracellular calcium, and release of arachidonic acid in response to activation of cystolic phospholipase A_2 ($cPLA_2$)[9, 10]. $cPLA_2$ activation in GEC was shown to disrupt endoplasmic reticulum (ER) membrane integrity and increased ER stress protein expression and their leakage into the cytosol. Inhibiting $cPLA_2$ attenuated complement-mediated cytotoxicity in GEC. In addition, inducing mild ER stress before induction of PHN showed a protective effect and was shown to reduce proteinuria,

potentially because ER stress proteins were allowed to build up in advance and help reduce the totality of the ER stress when the main insult occurred [11]. C5b-9 also activates mechanisms that restrict injury or facilitate recovery [11, 12].

Nephrotic Syndrome

Among the many functions of the glomerulus, the important functions include filtration of water and solutes, while at the same time restriction of the passage of proteins through the glomerular capillary wall (termed glomerular permselectivity). As alluded to earlier, the protein nephrin is the principal component of the filtration slit diaphragm. The latter spans the podocyte foot processes, and plays an integral role in maintenance of glomerular permselectivity. Nephrin, the NPHS1 gene product, is a 1241-residue transmembrane protein of the immunoglobulin family of cell adhesion molecules [8]. Nephrin interacts with other nephrin proteins from adjacent foot processes at their 35 nm extracellular domains to form the tight filtration structure at the middle of the slit [13]. A mutated nephrin gene results in congenital nephrotic syndrome of the Finnish type [8]. In mice, knocking-out the nephrin gene causes proteinuria and an absence of slit diaphragms, leading to neonatal death [14]. Nephrin requires proper N-glycosylation for folding in the ER before it reaches the plasma membrane [15], but some nephrin missense mutations do not transport to the cell surface, accumulate in the ER, and undergo ERAD [16, 17]. For this reason it is obvious that proper conformation and transport out of the ER to the cell surface are necessary to ensure the maintenance of the slit diaphragm.

Endoplasmic Reticulum Function and Protein Processing

The ER plays an important role in normal glomerular cell function and in injury. As one of the largest intracellular organelles, the ER is a crucial component of the secretory pathway, where nascent secretory proteins undergo folding, processing and quality control before being exported[18]. Additional responsibilities of the ER include synthesis of cholesterol, steroids and other lipids, as well as intracellular storage of calcium [19].

Any disruption of the ER folding process leads to activation of the ER quality control

(ERQC) mechanism. The ERQC mechanism improves folding and/or degradation of misfolded proteins by upregulating ER chaperones and slowing down overall protein translation. Some molecular chaperones include the classical glucose-regulated proteins (GRP), for example Hsp70 homolog, GRP 78 (BiP), or nonclassical lectin-like chaperones, such as calnexin (CNX) and calreticulin (CRT), and various other chaperones [18, 19].

Proteins translocated into the ER are most commonly glycoproteins. Presynthesized oligosaccharides are added to the nascent proteins *en bloc* in the lumen of the ER as the protein enters the ER through the aqueous channel formed by translocon Sec61 [20]. These sugar moieties consist of three glucoses, nine mannoses and two N-acetylglucosamines ($Glc_3Man_9GlcNAc_2$)[21]. This transfer of the N-glycan to the polypeptide is catalyzed by an eight subunit protein called translocon-associated oligosaccharyltransferase. The glycan attaches specifically onto asparagine side chains within Asn-X-Ser/Thr sequences [22]. Correct N-linked glycosylation is crucial to proper secretory protein folding, quality control, sorting, degradation and secretion. The addition of these sugar moieties stabilizes proteins in the process of folding, and acts as recognition tags allowing interactions with lectins, glycosidases and glycosyltranferases [21]. This sugar tag renders the glycoprotein highly hydrophilic, leading to the outermost glucose moieties being immediately cleaved off by glucosidase I and II (Glc I, Glc II). This allows certain glycan-dependent chaperones, calnexin/calreticulin (CNX/CRT) to associate with the now monoglucosylated glycoprotein for maturation [20, 23]. Removal of the last glucose by Glc II results in a loss of affinity for CNX/CRT. However, if there are exposed hydrophobic patches remaining (meaning the protein is not yet properly folded), the protein is transferred to classical chaperones or enters a second CNX/CRT folding cycle with the help of UDP-Glc glycoprotein glycosyltransferase.

Misfolded proteins lacking proper N-linkages are retained in the ER, sometimes aggregating non-covalently with BiP or covalently to each other through disulfide bonds. The classical chaperones are involved in binding unfolded proteins to help with

degradation. The nonclassical chaperones allow entry into the CNX cycle, which is also thought to be involved in retaining misfolded proteins in the ER through interaction of monoglucosylated high mannose oligosaccharides to CNX's lectin domain. Another component of the ERQC involves ER mannosidases, which affects the retention time of misfolded glycoproteins on the ER chaperones. These molecular chaperones, mannosidases and other response elements work together to allow misfolded proteins multiple chances for proper folding, but the longer they stay in the ER, the higher the chance they are targeted for degradation. However, if after several attempts, or if an accumulation of misfolded proteins occurs, the ER communicates with the nucleus to respond through several signaling mechanisms to modulate protein expression and protect the cell from ER stress [18, 20].

ER stress can be caused by a vast variety of causes, including, but not limited to, changes in the homeostasis of calcium, glucose and ATP levels, the redox environment, availability of nutrients or oxygen, enhanced protein synthesis or insufficient post-translational modifications, etc. [24]. Cells in an ER stressed state, whether physiological or pathological, become less efficient at protein folding, which results in an accumulation of misfolded proteins retained in the ER lumen. In order to rescue these misfolded proteins, two main quality control mechanisms become activated: the unfolded protein response (UPR) and ER-associated degradation (ERAD)[19].

Unfolded Protein Response

Three main protein sensors: activating transcription factor-6 (ATF6), inositol-requiring enzyme 1α (IRE1α) and PKR-like ER kinase (PERK), are responsible for the activation of the UPR signaling pathway. In an unstressed state, the three proteins associate with ER chaperone, BiP. However, upon accumulation of misfolded proteins, BiP, which has a higher affinity for misfolded proteins, allows the three sensors to be released and become activated [19].

IRE1, the most conserved of the UPR activators, is a type I transmembrane protein with a serine/threonine kinase domain and an endoribonuclease (RNase) domain on the cytosolic side of the protein. Two homologs of IRE1 exist: IRE1α, expressed ubiquitously, and IRE1β, which is expressed exclusively in the intestinal epithelium. Upon IRE1α release from BiP, IRE1α trans-autophosphorylates and dimerizes, resulting in activation of its RNase activity. The cytosolic endoribonuclease domain of IRE1α cleaves a 26-base intron from X-box-binding protein-1 (XBP1) mRNA, resulting in a translational frame shift, thereby creating a basic leucine zipper transcription factor. XBP1 activates the transcription of target genes through binding to promoters of the UPR element and ER stress-response elements, I and II. Thus, XBP1 induces the expression of various genes that work together to enhance ER protein folding, secretion, quality control, ERAD, and activation of phospholipid biosynthesis, as well as genes that cause the ER to expand during ER stress. Lastly, IRE1 reduces protein synthesis through its stress-dependent degradation of specific mRNAs, termed regulated IRE1-dependent decay of mRNAs [25].

ATF6 is a type II ER transmembrane protein. Upon disassociation from BiP, ATF6 is able to enter the Golgi apparatus, where its transmembrane site is cleaved by site-1 and site-2 proteases. The now cleaved cytosolic fragment of ATF6 migrates to the nucleus to activate gene transcription of ER chaperones. ATF6α has several functions, including transactivation of certain ER chaperones, such as BiP, and heterodimerization with XBP-1 to induce the expression of proteins that promote ERAD. ATF6α is necessary for protein folding, secretion and degradation during ER stress [25].

PERK is a type I transmembrane protein that contains a cystolic serine/threonine kinase domain. Upon release from BiP, PERK undergoes trans-autophosphorylation and oligomerization. Activated PERK inhibits eukaryotic translation initiation factor-2α (eIF2α) through phosphorylation. Phospho-eIF2α causes global translational suppression, which helps reduce the ER protein-folding load during ER stress. Phospho-eIF2α is also essential for expression of several UPR genes, including activating transcription factor 4 (ATF4), which promotes the transcription of ER chaperone genes encoding BiP and GRP94, UPR-associated transcription factors, XBP1, and intracellular trafficking machinery that

promotes movement of ATF6 from the ER to the Golgi during ER stress [25]. Additionally, ATF4 is involved in cellular homeostasis, amino acid metabolism and redox reactions [26].

ER Associated Degradation

In physiological situations, ERAD is maintained at a baseline rate to avoid interfering with regular transcription/translation functioning. However, this limited ERAD capacity still has an important function to degrade terminally misfolded or mutated proteins. Specific ER chaperones recognize misfolded proteins through detection of hydrophobic regions, unpaired cysteines, and immature glycans. ERAD substrates interact with distinct chaperones, which is dependent on where the substrate is located, for example, ER membrane, ER lumen, or in the cytoplasm [27]. As previously mentioned, misfolded glycoproteins may pass through a second attempt of the CNX/CRT chaperone system. If this does not correct the misfolding, those glycoproteins go through a proposed "mannose timer" model, which explains that sequential mannose trimming of N-glycans removes those proteins from being able to interact with CNX/CRT, thereby removing them from further folding attempts [21, 28, 29]. This mannose trimming occurs through ER mannosidases, resulting in an α1,6-linked mannose-containing oligosaccharide signal, which is a required step for entry into and recognition by ERAD machinery [30]. The glycoprotein then associates with ER lectins such as EDEM, a mannosidase, and OS-9 and XTP3-B, targeting substrates to an ERQC compartment. As a result, ER function changes from primarily pro-folding, to pro-degradation [20].

ERAD substrates exit the ER when they have a strong exit signal; for example, when most of their α1,2 mannose residues have been removed. Through OS-9 and XTP3-B, the proteins destined for degradation associate with membrane-anchored HRD1-SEL1L, an ubiquitin ligase complex. This enables substrates to be retrotranslocated and simultaneously ubiquitinated as they move into the cytosol to be degraded by the 26S proteasome [29].

Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) is a component of ERAD and the final step in the degradation process of proteins not only from the ER, but also the cytosol and nucleus. The

UPS substrates consist of misfolded proteins, short-lived proteins, unfolded proteins, aberrantly modified proteins, etc. If these proteins are not degraded in a timely fashion, they can aggregate and accumulate. This aggregation contributes to the cause of 'conformational diseases', while the accumulation backs up ERAD machinery, which can further amplify ER stress. It is thought that around one-third of all proteins are degraded shortly after synthesis, although the reasons for this are still unclear [31]. This implies that the proteasomal degradation machinery is working at a significant capacity in normal physiological conditions, and must work even harder when the UPS is enhanced under ERAD.

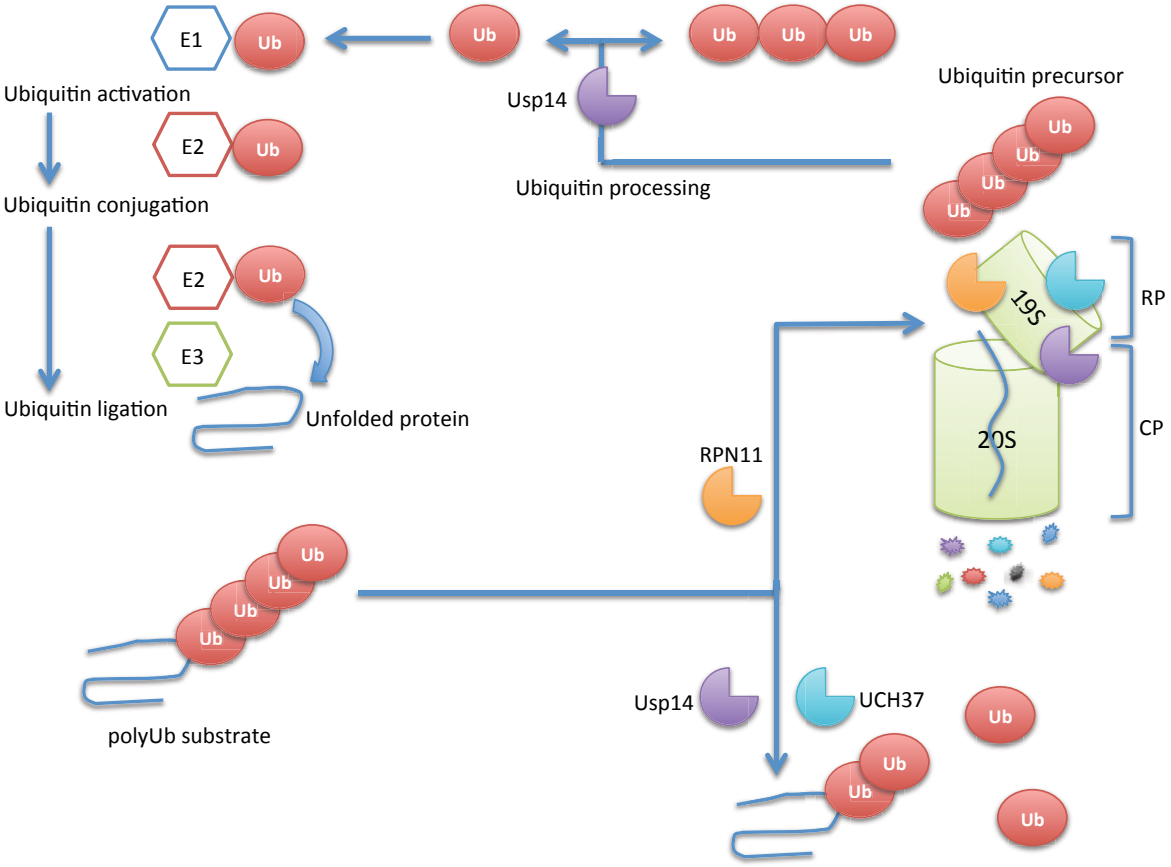
The UPS resides in both the cytosol and the nucleus, and is capable of degrading virtually any polypeptide chain through the chymotrypsin-like, trypsin-like and caspase-like activities found in the barrel-shaped complex, called the proteasome. The 26S proteasome complex is made up of a proteolytic 20S core particle, and two 19S regulatory particles, which cap and safeguard the core from non-specific protein degradation [31]. Degradation specificity for proteins is marked by covalent linkage of a chain of ubiquitin proteins added through a cascade of ubiquitin conjugating enzymes. This cascade includes: ubiquitin activase (E1), one of several ubiquitin conjugases (E2s), and one of many different ubiquitin ligases (E3s) (Fig. A). This results in the substrate being linked via the ε-NH_2 group of its internal lysine to the C terminus of the 76 amino acid ubiquitin polypeptide. Ubiquitin contains seven lysine residues allowing for successive ubiquitylation, resulting in the formation of polyubiquitin chains [31]. There are two main lysine residues used in ubiquitin linkage: K-48 and K-63 linkage. K-48 linkage is thought to be in involved mainly in targeting the protein to the proteasome, while the K-63 linkage is thought to be proteasome-independent, and mostly involved in signaling pathways [32], although recent studies have suggested a role for K63 linkage in ubiquitin-dependent proteasomal degradation as well [33-35]. Once polyubiquitinated by chains of four or more ubiquitin moieties, proteins can bind to the 26S proteasome, or to local shuttling factors through specific ubiquitin-binding domains (UBDs). However, ubiquitin must be effectively cleaved off just before the protein can enter the narrow entrance of the proteasome for hydrolysis [31].

Deubiquitinating Enzymes

Deubiquitinating enzymes (DUBs) consist of a large group of enzymes that cleave ubiquitin off ubiquitin-linked molecules. Specifically, the cleavage occurs at the terminal carbonyl of the last residue of ubiquitin (glycine 76)[32]. There are a variety of different DUBs, which fall into specific categories depending on their sequence homology and likely mechanisms of action. Generally speaking, most DUBs essentially target the same or similar goals; the removal of ubiquitin allows for effective polypeptide entry into the proteasome, and cleavage of polyubiquitin chains into single monomers helps recycle the free ubiquitin pool (Fig. A). Ubiquitin precursors have C-terminally extended domains, which must be properly processed for the generation of conjugation-competent ubiquitin. Several DUBs can cleave this C-terminal extension, either post- or co-translationally, suggesting that some DUBs are more efficient or abundant than others [36].

Protein degradation is a highly specific process. DUBs help maintain this specificity by balancing the addition and removal of ubiquitin chains to the protein substrate. If a protein substrate targeted for degradation - but not yet committed to entering the proteasome - encounters a DUB, which cleaves off several ubiquitin moieties, this may result in that protein substrate escaping degradation. Conversely, a protein-degradation substrate that has been around longer may be highly polyubiquitinated and hence not influenced by light chain trimming activity. This ensures a form of seniority, and prevents some inappropriately ubiquitinated substrates from being degraded, while promoting the degradation of specifically-targeted proteasome substrates [36]. A second way that protein degradation may be a specific process is that certain DUBs would deubiquitinate only certain classes of proteins, and not affect others. This method of specificity and its degree of importance is still uncertain and warrants further investigation.

Figure A: Ubiquitin processing pathway



Proteasome-Associated Deubiquitinating Enzymes

One of the main functions of DUBs is to remove ubiquitin chains from ubiquitin-protein conjugates once they are committed to the proteasome to be degraded. Removal of ubiquitin allows the protein to be unfolded properly before entering the narrow chamber of the 20S proteasome; ubiquitin is then cleaved into monomers and therefore recycled into the cytosol's free ubiquitin pool [36]. Three DUBs are known to be associated with the mammalian 26S proteasome: Regulatory proteasomal subunit 11 (Rpn11), ubiquitin C-terminal hydrolase 37 (Uch37) and ubiquitin-specific protease 14 (Usp14) [37, 38]. Rpn11 is a stoichiometric subunit of the lid subcomplex of the 19S proteasome [39, 40], while Uch37 and Usp14 associate reversibly [41]. Rpn11 removes polyubiquitin chains from proteins from the proximal end, thereby removing the whole chain at once *en bloc*. This function of Rpn11 has been shown to be essential in yeast *Saccharomyces cerevisiae* for

normal growth [42]. Uch37 and Usp14 remove ubiquitin from the distal end and Usp14 has also been shown to disassemble unanchored ubiquitin oligomers (Fig. A) [36, 38]. Uch37 is a member of the UCH family, Rpn11 of the MPN+/JAMM domain metalloproteases and Usp14/Ubp6, a member of the ubiquitin-specific processing proteases (UBPs) family [36].

Ubiquitin-Specific Protease 14

UBPs, the largest DUB family, are cysteine proteases with highly divergent sequences and have catalytic Cys and His residues that show strong homology. Human Usp14 contains 494 amino acids with a 9-kDa ubiquitin-like (Ubl) domain at its N-terminus followed by a 45-kDa catalytic domain. The structure and function of the catalytic domain is highly conserved among other representative UBPs, including with its yeast homolog, Ub6, with which it shares 31% sequence identity of the catalytic core domain [43]. The catalytic domain resembles an extended right hand consisting of three domains: Fingers, Palm and Thumb. This organization of domains creates a surface to which ubiquitin is thought to bind. Curiously, two surface loops named blocking loops 1 and 2 hover above the sides of the proposed binding pocket for the C-terminus of ubiquitin [43]. Interestingly both Uch37 and Usp14 are found in significant populations unassociated with the proteasome, but their catalytic functions increase upon binding to the proteasome [38]. This is seemingly odd since the catalytic domain is in the proper conformation regardless of its association with the proteasome [43]. However, the same researchers who crystalized the structure of the catalytic domain, showed that the two previously mentioned surface blocking loops 1 and 2, can undergo a drastic conformational change, effectively moving them away and allowing for a significantly wider binding groove for the C-terminus of ubiquitin to bind to. This supports the observation that proteasomal-association of Usp14 enhances its deubiquitinating activity [43].

As previously mentioned, Uch37 and Usp14 both remove ubiquitin from the distal end; the former removes monoubiquitin from chains, while the latter removes di- and triubiquitin from Lys48-linked conjugates [41, 43]. Thus, through shortening the ubiquitin chain, the substrate's affinity to the proteasome is reduced and this is thought to contribute to

rescuing proteins targeted to the proteasome to escape from degradation (Fig. A). This might be an important mechanism in the form of an editing process. The monoubiquitin cleavage mechanism of action of Uch37 can selectively suppress the breakdown of lightly ubiquitinated proteins, while leaving heavily ubiquitinated proteins to be degraded despite light trimming. This editing could help accelerate degradation of proper substrates, while inhibiting the degradation of incorrectly ubiquitinated substrates. However, the more recent proposal is that Uch37, and potentially more importantly, Usp14, due to its multiubiquitin cleavage activity, negatively regulate the degradation of proper substrates [41]. Recent findings have suggested that Uch37 and Usp14 can function redundantly to a degree. Knockdown of either protein resulted in enhancement of proteasome-dependent proteolysis and a decreased free ubiquitin pool [38].

Chapter 2: Hypothesis and aim of the study

In order that homeostasis be maintained in a healthy organism, specific and correct information must be transmitted within and amongst cells. When homeostasis is disrupted, disease ensues. The main workers of cell are the proteins, which are responsible for delivering signals around the cell in a timely and undisrupted fashion. Many different proteins are needed to supply the demand of proteins required in the nucleus, the membranes, the cytosol or to deliver messages to other cells. The high demand for proteins required intracellularly and extracellularly puts stress on protein biogenesis, which may result in errors in translation or the disruption of proper folding of proteins. As one of the major sites of protein maturation, the ER can come under stress when outside factors disrupt the internal homeostasis of the cell. As discussed, the UPR and ERAD are adaptive mechanism by which the cell responds to ER stress, and activation of the UPR and ERAD is designed to rescue the cell from long-lasting injury. ERAD attempts to get rid of the excess of misfolded or aberrant proteins by retrotranslocating them to the cytosol for degradation by ubiquitin-proteasomal machinery.

A broad spectrum of external insults can disrupt the internal homeostasis of a cell. The complement C5b-9 membrane attack complex is one such insult, and it plays a role in the development of certain glomerular diseases, specifically membranous nephropathy, a major cause of idiopathic nephrotic syndrome in adults [44]. The complement-mediated attack on the podocyte results in increased expression of ER stress proteins and can contribute to injury of the podocyte [11, 45]. Complement activation stimulates the UPS and increases protein ubiquitination, and inhibiting or delaying proteasome functioning exacerbates complement cytotoxicity [12]. In this study, we propose to investigate a method for enhancing UPS function, including proteasomal degradation of misfolded proteins, with the purpose of ameliorating ER stress and preventing cellular injury upon complement activation.

Unlike Rpn11, Usp14 deubiquitinates proteasomal substrates before they are committed to degradation, thereby rescuing the protein from degradation. It was shown that yeast

homolog Ubp6 was a potent inhibitor of the proteasome through its catalytic (deubiquitinating) activity, but also through a non-catalytic delay of degradation, that is poorly described [46]. Overexpression of Usp14 delayed the degradation of several ubiquitin-protein conjugates, both *in vitro* and in cells. Contrastingly, in the presence of a small-molecule inhibitor of Usp14's deubiquitinating activity, the degradation of several proteasomal substrates involved in neurodegenerative diseases was enhanced. Inhibiting Usp14 also seemed to protect against oxidative stress. Interestingly, a catalytically inactive mutant of Usp14 seemed to inhibit the proteasome, but to a lesser degree than the wild type Usp14, suggesting that Usp14 may also contains the peculiar non-catalytic proteasomal inhibition [47]. It was also shown that Usp14 interacts with the cytosolic fragment of IRE1α in physiological states, and ER stress disrupts this interaction. Overexpressing Usp14 inhibits ERAD and knocking down Usp14 activated ERAD [48]. We propose that inhibition of Usp14 in a stressed cell, such as after complement activation or after exposure to other ER stressors, will enhance the degradation of proteasomal substrates, thereby preventing misfolded protein accumulation and podocyte injury. While our studies are directed towards the pathogenesis of glomerulonephritis, further elucidation of the link between ERAD and UPS is important for understanding the mechanisms of other ER stress-mediated diseases.

In the present study, we employed GEC and HEK 293T (293T) cells with Usp14 overexpression or inhibition of catalytic activity by small molecule inhibitor, IU1. To test the proposed hypothesis, cells were stimulated by complement to investigate how this affects the performance of ERAD and UPS. To assess the functionality of the UPS and ERAD, an UPS reporter, GFP^{u} (CL1 degron fused with green fluorescent protein), and an ERAD reporter, CD3δ-yellow fluorescent protein (YFP), was employed, respectively. Similar investigations were carried out with endogenous substrates, such as nephrin, to assess this comparison in more physiologically relevant models. In addition, complement-mediated and other ER stress inducing cytotoxicity was studied to investigate if IU1 can be employed to enhance cytoprotection.

Chapter 3: Materials and Methods

Reagents

Cell culture supplies, pRc RSV DNA, fetal bovine serum, DMEM, transfection reagents, Lipofectamine 2000 and OptiMEM were purchased from Invitrogen Life Technologies (Burlington, ON) and Wisent (Saint-Jean Baptiste, QC). NuSerum was purchased from BD Bioscience (Bedford, MA). Cycloheximide, MG132, tunicamycin, DMEM, mouse anti-tubulin antibody, and rabbit anti-actin antibody were obtained from Sigma-Aldrich Canada (Mississauga, ON). Electrophoresis and immunoblotting reagents were purchased from Bio-Rad Laboratories (Mississauga, ON). Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagents were purchased from Amersham GE Healthcare (Baie d'Urfé, QC). Bradford Reagent was purchased from Fermentas (Burlington, ON). Plasmid $pGFP^u$ was kindly provided by Dr. Ron Kopito (Stanford University, Stanford, CA)[49]. CD3δ-YFP cDNA was purchased from Addgene and was described previously [50]. Usp14 and Usp14C114A DNA were kindly provided by Dr. Daniel Finley (Harvard University, Boston, MA)[47] Nephrin mutant I171N and S724C DNAs were kindly provided by Dr. Karl Tryggvason (Karolinska Institutet, Stockholm, Sweden). Mouse anti-green fluorescent protein (GFP) and mouse anti-ubiquitin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Usp14 from Bethyl (Montgomery, TX), and rabbit anti-nephrin (which reacts with the cytoplasmic domain of nephrin) was kindly provided by Dr. Tomoko Takano (McGill University)[51]. Secondary antibodies include peroxidase-conjugated AffiniPure sheep anti-mouse IgG (H+L), goat anti-rabbit IgG (H+L) from Jackson ImmunoResearch (West Grove, PA). Production of rabbit anti-rat GEC antiserum was described previously [9]. 1-[1-(4-Fluorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-(1-pyrrolidinyl)ethanone (IU1) was used as a specific, reversible inhibitor of the catalytic site of deubiquitinating enzyme, Usp14, was purchased from Cayman Chemical (Ann Arbor, MI)[47].

Cell Culture

Human Embryonic Kidney 293T cells (293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Primary cultures of GEC were established from explants of rat glomeruli, and have been characterized previously [52, 53]. Rat GEC were maintained in a 1:1 ratio of DMEM to Ham's F12 medium, supplemented with 5% NuSerum and 0.5% hormone mix. NuSerum contains 25% fetal bovine serum and 5 ng/ml epidermal growth factor and hormones. Hormone mix contains insulin (5 µg/ml), prostaglandin E1 (25 ng/ml), triiodothyronine (0.325 ng/ml), Na_2SeO_3 (1.73 ng/ml), apo transferrin (5 µg/ml), hydrocortisone (18.12 ng/ml) in DMEM. All cell cultures were maintained at 37˚C in a mix of 5% CO_2 and 95% air. Confluent cells were passed by rinsing with Ca^{2+} and Mg^{2+} free Hank's balanced salt solution prior to incubating with 0.05% trypsin-0.02% EDTA in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution until cells detached. Cells were then re-plated in a 1/10 dilution. All cell lines used in experiments were between passages 25 and 80.

Transfection and Cell Harvesting

Upon being trypsinized, cells were counted using a hemocytometer. 2.5 x 10^5 293T cells and GEC were passed into 60 mm plates and incubated for 24 – 48 h. Cells were transiently transfected at 70-90% confluency with Lipofectamine 2000 and DNA according to the procedure provided by the manufacturer (2:1 ratio, respectively). Plasmids that were transfected consisted of: CD3δ-YFP, GFP^u, pRc/RSV, Usp14, Usp14 C114A, nephrin mutant I171N or nephrin mutant S724C. The transfection was performed by first creating a master mix of Lipofectamine 2000 and OptiMEM, which was incubated for 5 min at 22°C. The master mix was then distributed to each plasmid DNA sample in OptiMEM, which was incubated for 20 min 22°C. This solution was then added directly onto the cells. Transfected cells were rinsed two times with PBS and lysed with 100 µl-200 µl of lysis buffer (1% Triton X-100, 125 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EGTA, 2 mM Na_3VO_4, 5 mM $Na_4P_3O_7$, 25 mM NaF and 10 µl/ml inhibitor cocktail from Bioshop Canada (Burlington, ON). Lysates were centrifuged for 10 minutes at 10,000g.

Immunoblotting

Protein concentrations were measured via a Bradford assay and standardized to prepare equal amounts of protein for loading into the gel. Laemmli sample buffer was added to the lysis buffer and boiled for 1 h. Proteins were separated by SDS-PAGE under reducing conditions, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin in 1X TTBS (containing 20 mM Tris 137 mM NaCl, 0.1% Tween 20, pH 7.6). Membranes were incubated with a primary antibody overnight at 4°C, washed three times in TTBS (5 minute washes), incubated with horseradish peroxidase labeled secondary antibody for 60 minutes at 22°C and washed three times with TTBS (5 minute washes). Following this, membranes were incubated with ECL reagent and exposed to X-ray film. The ECL signals were quantified by scanning densitometry. Immunoblot films were scanned and converted to negative images. Specific bands of interest were selected, and the density of the bands was measured using National Institutes of Health Image J software. Results are expressed in arbitrary units.

Measurement of Cytotoxicity

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) from the cytosol into cell supernatants. LDH converts lactate and NAD to pyruvate and NADH. The rate of increase in the absorbance of the reaction at 340 nm, due to the formation of NADH, represents LDH activity [52]. Cells were cultured in 15 mm wells for 48 h. Cells were then washed twice with measurement buffer (containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO4, 1 mM Na2HPO4, 5 mM glucose, 20 mM Hepes, 0.5 mM CaCl2) for 5 min at 37°C. After incubation with complement or other cell stressors such as, tunicamycin, supernatants from all wells were collected and 1% Triton X-100 solution was added to each well (to collect lysed cell extracts) for 5 min at 22°C while shaking. Upon collecting Triton X-100 solution from each well, supernatants and Triton X-100 samples (25 µl/well) were combined separately with working reagent (175 µl/well; 58 mM lithium lactate, 0.2 M glycine, and NAD) and were analyzed separately for LDH activity in duplicate in a 96-well plate on a plate reader set at 340 nm at 37°C.

After incubation of cells with or without tunicamycin (1 μg/ml, 5 μg/ml or 10 μg/ml), specific release of LDH was calculated as [tunicamycin – DMSO alone]/[100- DMSO alone], where tunicamycin represents the percent total LDH released into cell supernatants in incubations with tunicamycin, and DMSO alone represents the percent total LDH released into cell supernatants in incubations with DMSO (vehicle) alone as control.

Complement was activated by first incubating cells with 5% rabbit anti-rat GEC antiserum in measurement buffer for 40 min at 22°C. After the incubation period, the rabbit anti-rat GEC antiserum was removed and replaced with either 2.5%, 4%, 5%, 7%, 10% or 15% normal human serum (NS), or heat-inactivated serum (HIS; 56°C for 1 h) in controls, diluted in measurement buffer for acute incubations (40 mins at 37°C) or diluted in K1 medium for chronic incubations (4 h or 24 h at 37°C). In GEC, complement is not activated in the absence of antibody [52]. Specific release of LDH was calculated as [NS – HIS]/[100 – HIS], where NS represents the percent total LDH released into cell supernatants in incubations with NS, and HIS represents the percent total LDH released into cell supernatants in incubations with HIS.

Statistics

Results are presented as mean ± standard error of the mean (SEM). The t statistic was used to determine significant differences between two groups. One-way analysis of variance (ANOVA) was conducted to determine significant differences among three or more groups. Two-way analysis of variance was conducted to determine significance when there was more than one independent variable. Where significant differences were found, individual comparisons were made between groups using the t statistic, and adjusting the critical value according to the Bonferroni method. Statistical significance was considered at $p < 0.05$

Chapter 4: Results

Usp14 Regulates UPS Function in GEC

Certain glomerular diseases involving complement C5b-9 membrane attack complex formation, demonstrate accumulation of misfolded proteins, resulting in GEC injury and proteinuria, and increased expression of ER stress proteins [11]. In cultured cells, complement-mediated injury can be monitored by release of LDH, i.e. in dying cells, the plasma membrane integrity is compromised, and cytosolic LDH is released through the damaged cell membrane into the culture medium. The active site inhibitor of Usp14, IU1, was shown to enhance the degradation of certain misfolded proteins known to aggregate in conformational diseases [47]. The first set of experiments was designed to establish the concentration of IU1, which would not be toxic to cells in the context of C5b-9 assembly. GEC were pretreated with IU1 at varying concentrations (50 μM, 75 μM and 100 μM) and were incubated with anti-GEC antiserum for 40 min, and then NS (2.5% or 5%) to form C5b-9, or HIS (2.5% or 5%) in controls for either 4 or 24 h. We tested the dose-response effects of IU1 at 2.5% NS, a "sublytic" dose of complement activity, which induces minimal cell death [12]. IU1 at 50 μM when administered with 2.5% NS was shown to be non-toxic, compared to the higher doses of IU1 (Fig. 1). Thus, the 50 μM dose was employed in further studies.

GFP^u is a reporter of UPS function and consists of a 16-amino-acid-degron, CL1, fused to the C-terminus of GFP. The CL1 degron is a mutated C-terminal bulky hydrophobic residue and acts as a misfolded protein; thus, after expression in mammalian cells by transfection, GFP^u is targeted for ubiquitin-dependent proteasomal degradation [54]. GEC were co-transfected with GFP^u cDNA and either; control vector (pRc RSV), wild-type Usp14 or catalytically inactive mutant Usp14C114A (Usp14CA)[47]. Overexpressed wild-type Usp14 was shown to significantly increase the levels of GFP^u (reflecting decreased proteasomal degradation of the reporter), compared to both control vector and Usp14CA at both 24 h and 48 h after transfection. Thus, the proteasome-associated DUB Usp14 delays the functioning of the UPS (Fig. 2).

The expression levels of Usp14 were monitored by immunoblotting with anti-Usp14 antibody. The apparent expression levels of ectopic Usp14 or Usp14CA appeared to be ~1.5-1.7-fold above endogenous (Fig. 2A, C, D and F). However, the transfection efficiency of GEC is relatively low (below 30%); therefore, in cells co-transfected with GFP^u and Usp14 or Usp14CA, expression of the ectopic Usp14 proteins is predicted to be substantially greater than the levels appearing on the immunoblots, which reflect expression in both transfected and non-transfected cells.

It was previously shown that activation of complement resulted in enhancement of the UPS, which was demonstrated by significantly enhancing the degradation of GFP^u [12]. Twenty-four hours after transfection, GEC were incubated with anti-GEC antibody and NS at a sublytic concentration (to assemble C5b-9), or HIS in controls. GFP^u levels decreased in cells treated with NS, but not with HIS (Fig. 3). Some cells were also treated with Usp14 active site inhibitor, IU1 (50 μM) for 4 h and 24 h. IU1 treatment further significantly decreased GFP^u levels in cells where complement was activated (Fig. 3). These results are consistent with previous results showing that complement activation alone enhances GFP^u degradation, and blocking Usp14 may further enhance the UPS by increasing the degradation of some misfolded proteins.

Effect of Usp14 on ERAD

ER stress and an impaired UPS are involved in the pathophysiology of conformational diseases. During ERAD, proteins are deglycosylated in the ER and interact with lectins prior to transit from the ER to the cytosol for ubiquitination and proteasomal degradation. ER stress was reported to have an inhibitory effect on UPS [50]. As has been previously demonstrated in our lab, GFP^u is a reporter of proteasomal degradation and ubiquitination in the cytosol and nucleus. CD3δ-YFP is a fusion protein of a T cell antigen receptor (TCR) subunit CD3δ and an YFP. When expressed in non-T cells lacking other TCR subunits, CD3δ undergoes ERAD[55]. Our lab previously showed that complement activation attenuated the degradation of CD3δ-YFP [12]. GEC were co-transfected with CD3δ-YFP and either pRc RSV, wild type Usp14 or Usp14CA. Overexpressed Usp14 tended to increase the levels of CD3δ-YFP compared to both control vector and Usp14CA at 24 h, although this increase did

not reach statistical significance (Fig. 4). We also treated GEC with IU1, with or without activation of complement. In contrast to our findings with GFP^u, CD3δ-YFP levels were found to be very similar in HIS (2.5%) treated cells and NS (2.5%) treated cells. When we added IU1 to block Usp14, we saw a slight, but insignificant decline in CD3δ-YFP levels with both HIS and NS treated cells (Fig. 5). This finding of CD3δ-YFP degradation being delayed with complement, in comparison to the opposite finding with GFP^u, suggests that there is a rate-limiting step in ERAD possibly in the retrotranslocation out into the cytosol for UPS degradation

Effect of Overexpression and Inhibition of Usp14, on Human Nephrin Mutants I171N and S724C in HEK 293T Cells

Several distinct mutations in the nephrin gene are responsible for congenital nephrotic syndrome in humans [17]. To complement our experiments with the ERAD reporter, CD3δ-YFP, we studied two missense nephrin mutants, I171N and S724C. It was previously shown that the I171N mutation results in a non-conservative amino acid substitution that prevents the trafficking of nephrin to the cell surface, while the S724C mutation (which lies in a spacer region), did not apparently hinder transport to the cell surface[16]. Previous studies in our lab showed that I171N shows increased ubiquitination and interacts strongly with calnexin, which suggests it is severely misfolded. S724C on the other hand is misfolded more mildly, but despite its apparent ability to express on the cell surface, is still substantially degraded. The ER accumulation of both mutants was also shown to activate the UPR, specifically the ATF6 branch, which is thought to be an adaptive and cytoprotective pathway, and both mutants were shown to be degraded through ERAD[56]. We transfected two human nephrin mutants, I171N and S724C into HEK 293T cells. In one set of experiments, cells previously transfected with either of the nephrin mutants were co-transfected with control vector (pRc RSV), or wild-type Usp14, and were then treated with cycloheximide to block protein synthesis. Nephrin mutant degradation was monitored over 4 and 24 h. Both nephrin mutants I171N (Fig. 6) and S724C (Fig. 7) were shown to have significantly enhanced expression at 4 h after cycloheximide treatment in the presence of Usp14 overexpression, indicating that Usp14 is delaying their degradation. Conversely, in cells treated with cycloheximide, IU1 did not enhance the degradation of both nephrin

mutant I171N (Fig. 8) or S724C (Fig. 9). The lack of enhancement of degradation with IU1 was similar to the result seen with CD3δ-YFP, and could reflect rate-limiting retrotranslocation of misfolded proteins from the ER to the cytosolic UPS machinery. Since the folding of ER proteins with transmembrane domains may depend not only on ER chaperones, but also on cytosolic chaperones, the effect of IU1 on the nephrin mutants was also examined in the presence of pifithrin-μ, a chemical inhibitor of the cytosolic chaperone, Hsp70 [57]. Pifithrin-μ did not affect the degradation of the nephrin mutants independently, nor together with IU1 (Fig. 8 and 9).

Effect of Usp14 Inhibitor, IU1, on Cytotoxicity in Glomerular Epithelial Cells

Upon establishing that a 50 μM dose of IU1 was not independently toxic at a sublytic concentration of complement, we assessed if IU1 protected against cytotoxicity at higher doses of complement (NS greater than 2.5%). GEC were pretreated with IU1 (50 μM) or medium alone for 30 min. C5b-9 assembly was induced by incubating cells with anti-GEC antiserum for 40 min and then adding normal serum (4-15%) with or without IU1 (50 μM), or heat-inactivated serum (10-15%) in controls for 40 min, 4 h or 24 h at 37°C. IU1 did not protect GEC from complement-induced cytotoxicity (Fig. 10).

Co-translational modification of proteins with an N-linked oligosaccharide added to the amino acid motif, Asn-X-Ser/Thr, is important for proper maturation of most proteins that translocate into the lumen of the ER [58]. Tunicamycin is a specific ER stressor, which induces protein misfolding and ER stress by inhibiting N-glycosylation. Tunicamycin has been shown to cause an increase in CD3δ-YFP levels in GEC, similar to the complement induced increase of the ERAD reporter, noted above, and with prolonged incubation time, tunicamycin can induce cytotoxicity [12]. Thus, we addressed the role of Usp14 in modulating tunicamycin-induced cytotoxicity. By analogy to the complement-cytotoxicity experiment, we tested GEC pretreated with IU1 (for inhibition of Usp14), and stimulated by varying concentrations of tunicamycin (1, 5 or 10 μg/μl). IU1 did not affect cytotoxicity at the low and high doses of tunicamycin treatment, but significantly reduced cytotoxicity at the intermediate dose (Fig. 11).

Usp14 Inhibitor IU1 did not Modify Global Ubiquitination of Proteins

Proteasome inhibition may be caused by defects in ubiquitin turnover [59]. Usp14 is one of several deubiquitinating enzymes associated with the 19S proteasome [37]. It has been previously shown that loss of two proteasome-associated DUBs (one being Usp14) may lead to accumulation of polyubiquitinated proteins and an inhibition of protein degradation, whereas the loss of just one DUB does not [38]. Similarly, inhibition of both DUBs by a small-molecule inhibitor showed the same above-mentioned phenotype and shifted polyubiquitinated proteins towards higher molecular weights [60]. We inhibited Usp14 in GEC with IU1 (50 μM) to examine ubiquitination of proteins. Some cells were treated with the proteasome inhibitor MG132 alone (to prevent proteasomal degradation of ubiquitinated proteins), IU1 alone, or both MG132 and IU1 together. Incubation of GEC with MG132 increased global protein ubiquitination, but co-incubation with IU1 did not enhance that increase. Inhibiting Usp14 alone did not significantly promote either a shift of proteins towards higher molecular weights, nor did it significantly increase global polyubiquitination of proteins (Fig. 12). This finding is consistent with the previous studies suggesting that proteasome-associated DUBs may display a degree of redundancy and supplant deubiquitinating activity of those that are inhibited. Alternatively, IU1 may be targeting only a limited subset of cellular ubiquitinated proteins, which is not detectable in the context of global protein ubiquitination.

Figures

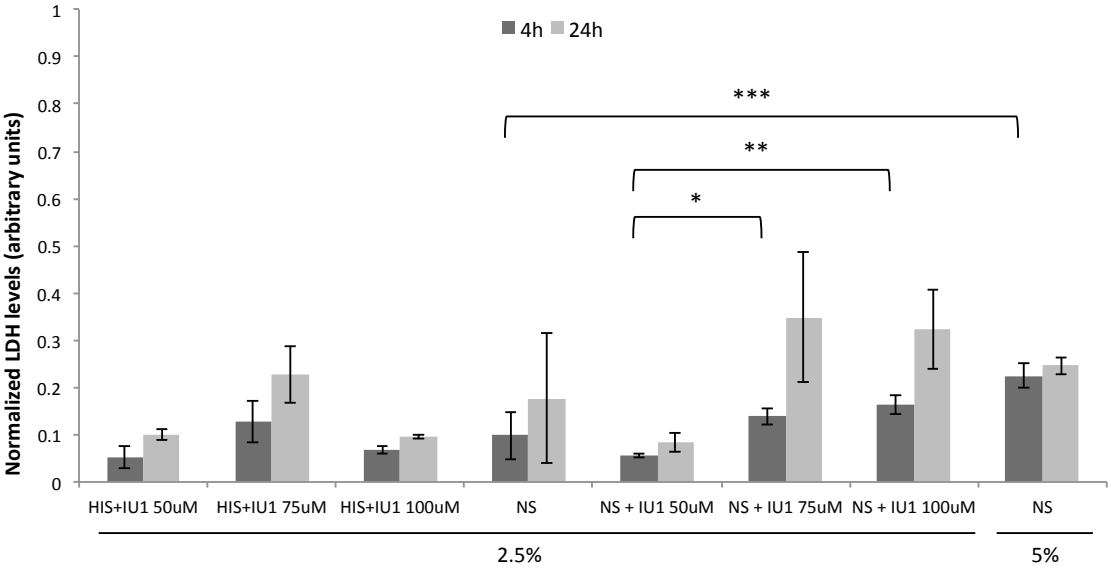


Fig. 1. Usp14 inhibitor, IU1, was not cytotoxic at a concentration of 50 μM, but cytotoxicity increased at 75 and 100 μM doses in the context of complement activation.

Cytotoxicity was assessed by LDH release after 4 and 24 h. GEC were pretreated with IU1 (50 μM, 75 μM or 100 μM) or medium alone for 30 min. C5b-9 assembly was induced by incubating all wells with anti-GEC antiserum for 40 min, and then adding normal serum (NS; to assemble C5b-9) at 2.5, or 5%, or heat-inactivated serum (HIS; control), together with IU1 (50, 75, or 100 μM) for 4 or 24 h. Cytotoxicity was monitored by a LDH assay, and was normalized to 2.5% or 5% HIS respectively. *$p<0.01$ (NS 2.5% + IU1 50 μM vs. NS 2.5% + IU1 75 μM at 4 h), **$p<0.05$ (NS 2.5% + IU1 50 μM vs. NS 2.5% + IU1 100 μM at 4 h and 24 h), ***$p<0.05$ (NS 2.5% vs. NS 5% at 4 h). N=5 experiments.

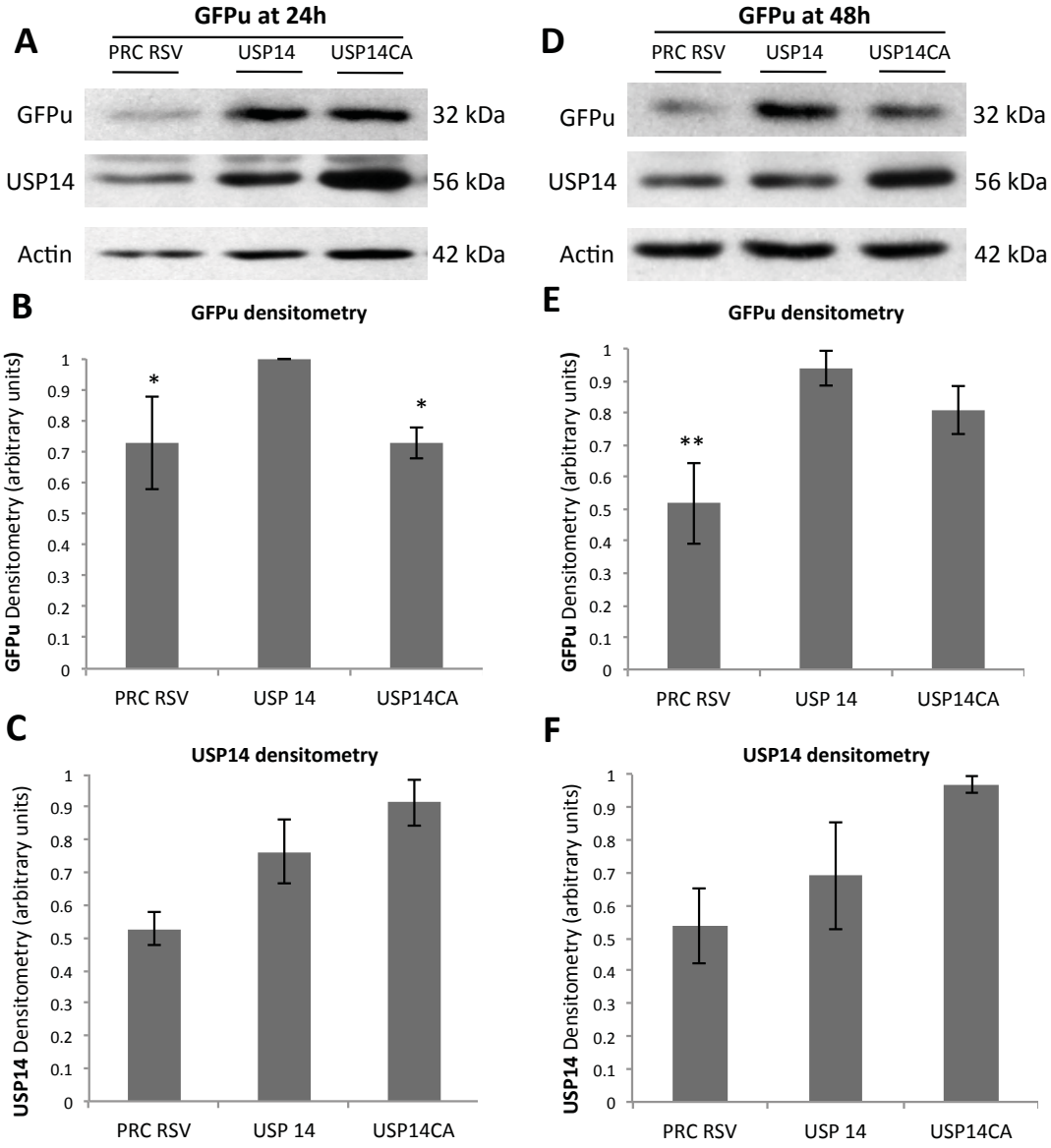


Fig. 2. Overexpression of Usp14 delayed the degradation of ubiquitin-proteasome system reporter, GFP^u, in glomerular epithelial cells.

GEC were co-transfected with GFP^u and either pRc RSV (control vector), Usp14 wild type or Usp14C114A (inactive mutant) for 24 or 48 h. Cell lysates were immunoblotted with antibodies to GFP, Usp14 and actin. (A and D) Representative immunoblots for GFP^u at 24 and 48 h after transfection, respectively. Densitometric quantification of GFP^u and Usp14 at 24 (B-C) and 48 h (E-F) after transfection. *p=0.05 GFP^u (Usp14 vs. pRc/RSV, Usp14 vs. Usp14CA), N=3 (24 h, B); **p<0.005 GFP^u (Usp14 vs. pRc/RSV), N=4 (48 h, E).

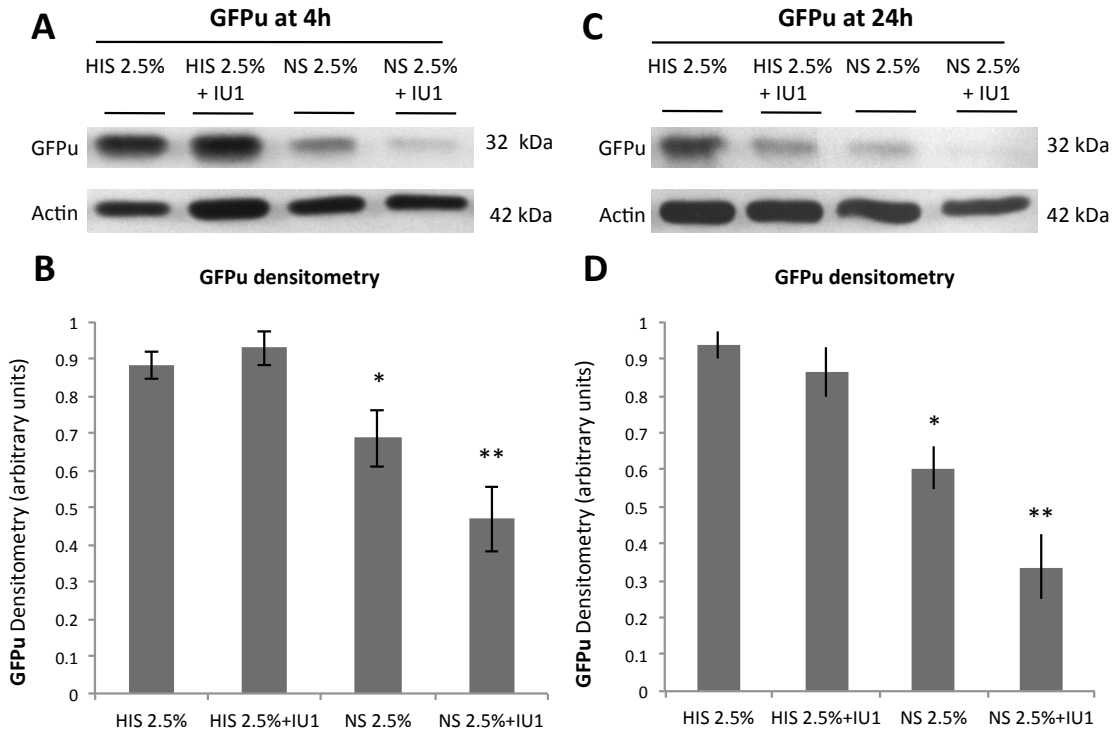


Fig. 3. Usp14 inhibitor, IU1, significantly increased the degradation of the UPS reporter, GFP^u, in complement-treated glomerular epithelial cells.

GEC were transfected with GFP^u. 24 h after transfection, cells were pretreated with IU1 (50 μM) or medium alone for 30 min. C5b-9 was assembled by incubating cells with anti-GEC antiserum for 40 min, and then with normal serum (2.5%) or heat-inactivated serum (2.5%), with or without IU1 (50 μM) for 4 (A and B) or 24 h (C and D). Cell lysates were immunoblotted with antibodies to GFP and actin. Representative immunoblots (A) and densitometric quantification of GFP^u (B and D) are presented. (B and D) *$p<0.05$ GFP^u (HIS vs. NS), N=6. **$p<0.05$ GFP^u (NS vs. NS+IU1), N=6.

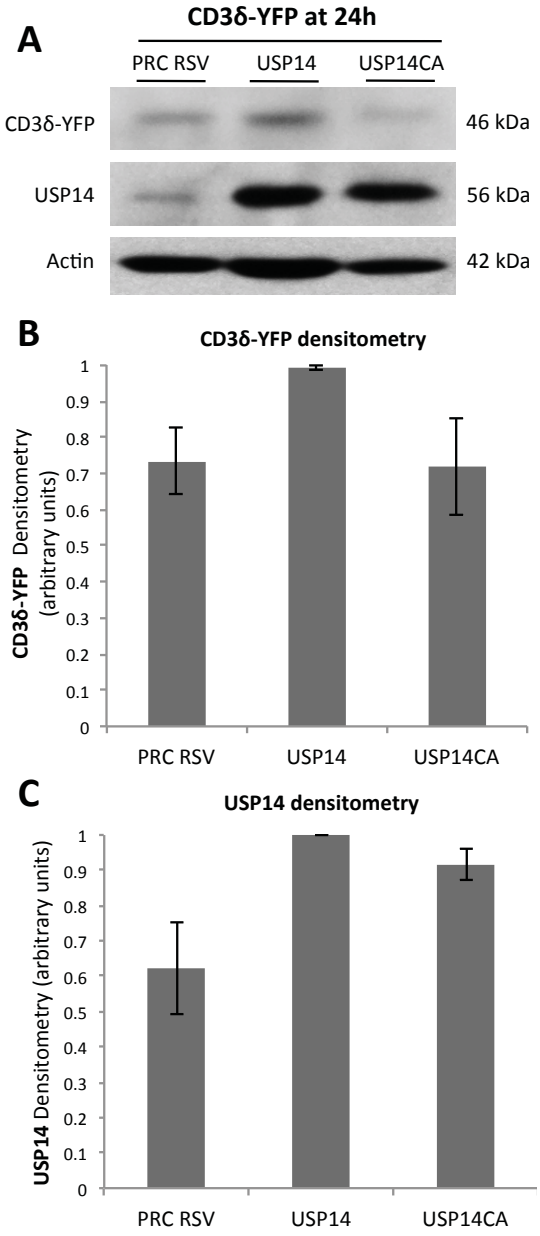


Fig. 4. Overexpression of Usp14 tended to delay degradation of the ERAD reporter, CD3δ-YFP, in glomerular epithelial cells.

GEC were co-transfected with CD3δ-YFP and either pRc RSV (control vector), Usp14 wild type or Usp14C114A (inactive mutant). After 24 h, cell lysates were immunoblotted with antibodies to GFP, Usp14 and actin. (A) Representative immunoblot of CD3δ-YFP 24 h after transfection. Densitometric quantification of CD3δ-YFP and Usp14 24 h after transfection (B and C). Differences between groups did not reach statistical significance. N=5.

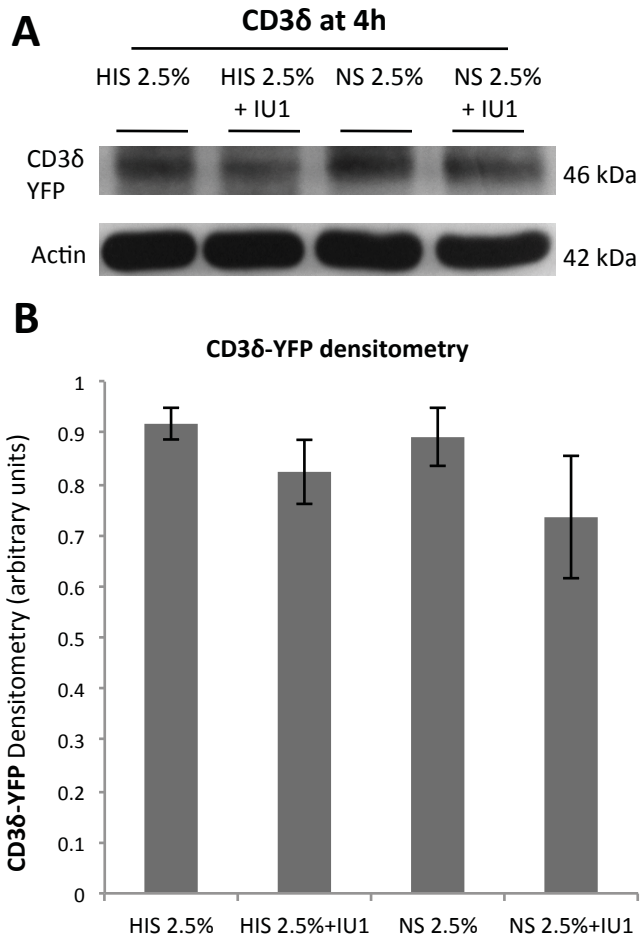


Fig. 5. Usp14 inhibitor, IU1, tended to increase the degradation of the ERAD reporter, CD3δ-YFP, in complement-treated glomerular epithelial cells.

GEC were transfected CD3δ-YFP. 24 h after transfection cells were pretreated with IU1 (50 μM) or medium alone for 30 min. C5b-9 was assembled by incubating cells with anti-GEC antiserum for 40 min, and then with normal serum (2.5%) or heat-inactivated serum (2.5%), with or without IU1 (50 μM) for 4 h. Cell lysates were immunoblotted with antibodies to GFP and actin. Representative immunoblots (A) and densitometric quantification of GFP (B) are presented. N=6.

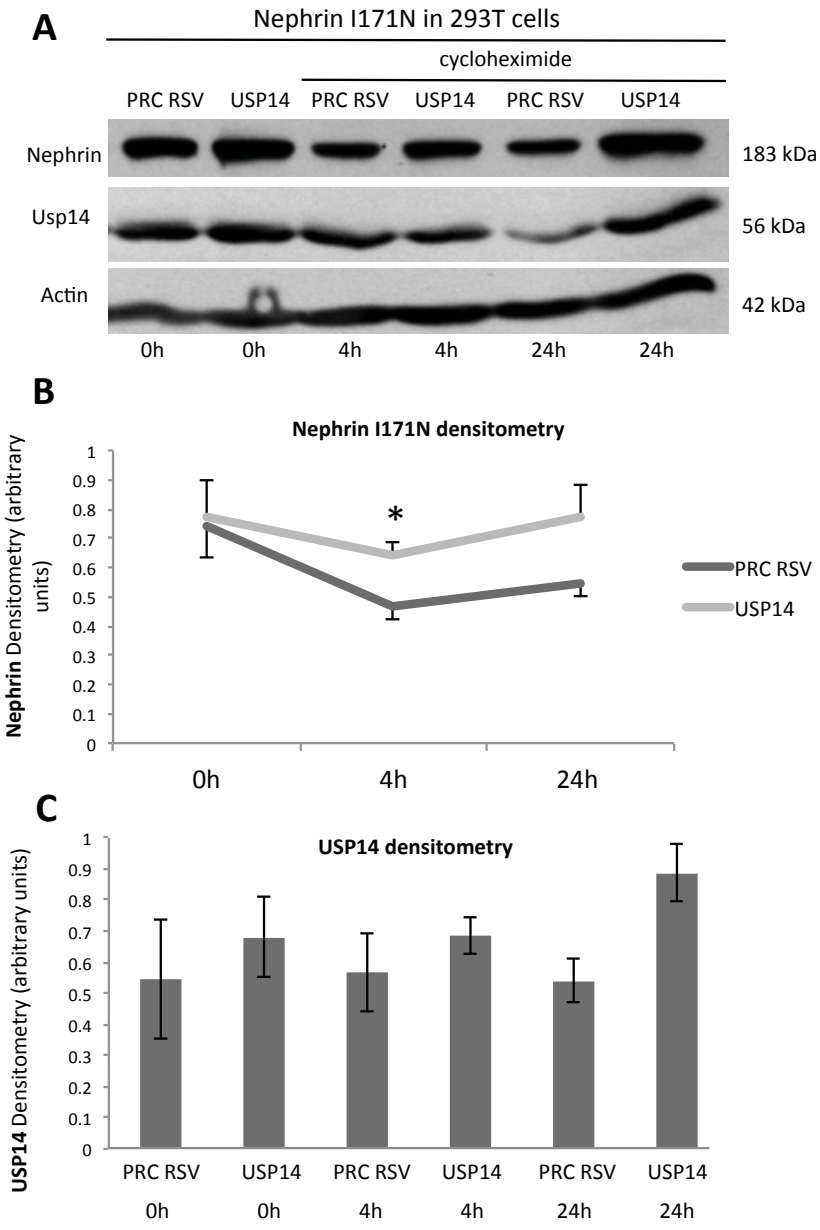


Fig. 6. Usp14 overexpression significantly decreased the degradation of the I171N nephrin mutant in HEK 293T cells.

HEK 293T cells were transfected with human nephrin mutant I171N. After 24 h, transfected cells were treated with cycloheximide (25 μM) to block protein synthesis and were lysed at 0, 4 or 24 h. Cell lysates were immunoblotted with antibodies to nephrin, Usp14 and actin. Representative immunoblot (A) for nephrin I171N, and densitometric quantification of nephrin (B) and Usp14 (C) are presented. *$p<0.05$ Nephrin (pRc RSV vs. Usp14 at 4 h), N = 3.

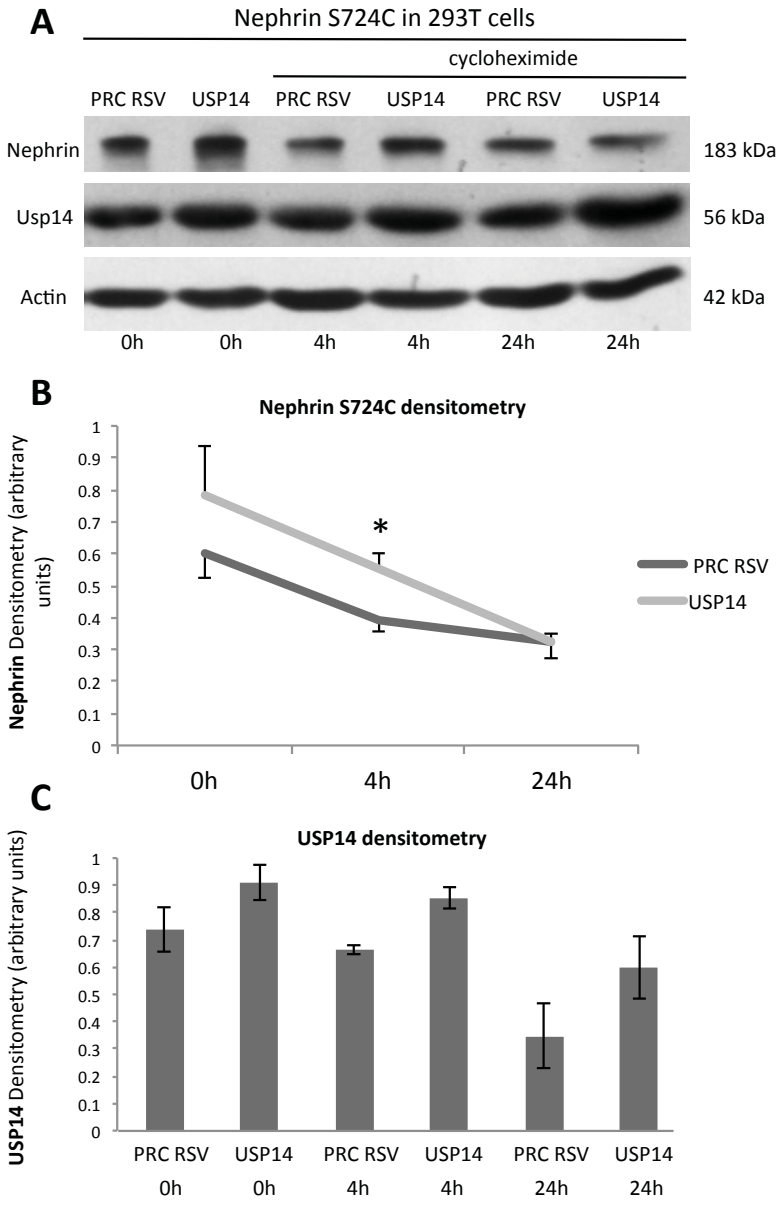


Fig. 7. Usp14 overexpression significantly decreased the degradation of the S724C nephrin mutant in HEK 293T cells.

HEK 293T cells were transfected with human nephrin mutant S724C. After 24 h, transfected cells were treated with cycloheximide (25 μM) to block protein synthesis and lysed at 0, 4 or 24 h. Cell lysates were immunoblotted with antibodies to nephrin, Usp14 and actin. Representative immunoblots for nephrin S724C (A), and densitometric quantification of nephrin (B) and Usp14 (C) are presented. *$p<0.05$ Nephrin (pRc RSV vs. Usp14 at 4 h), N = 6.

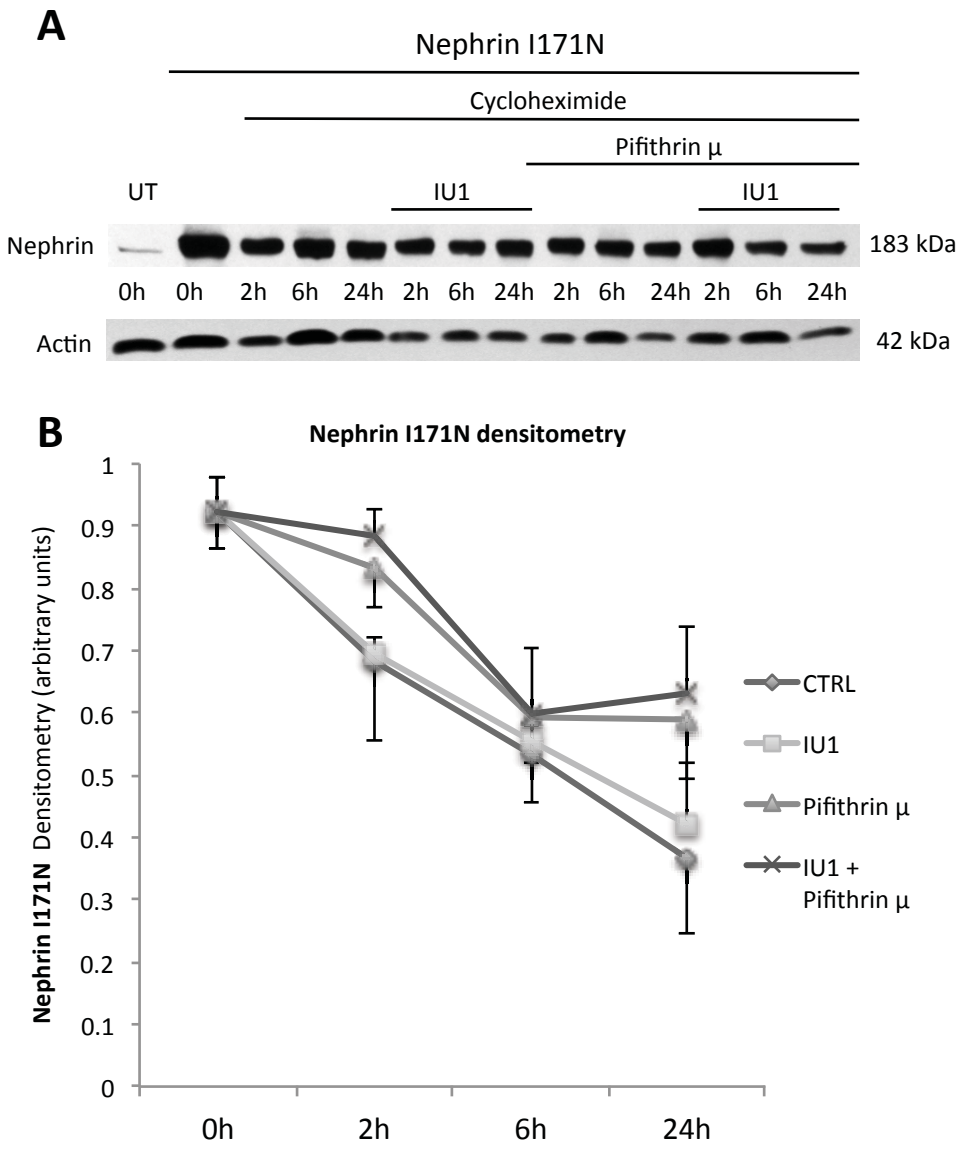


Fig. 8. Usp14 inhibitor, IU1, did not accelerate the degradation of nephrin mutant I171N in HEK 293T cells.

HEK 293T cells were transfected with human nephrin mutant I171N. After 48 h, transfected cells were treated with cycloheximide (to block protein synthesis), IU1 (50 μM), pifithrin μ (Hsp70 inhibitor; 5 μM), or with both IU1 and pifithrin μ. Each of these conditions was tested for at either 2, 6 or 24 h, with untransfected and untreated (0 h) controls. Cell lysates were immunoblotted with antibodies to nephrin and actin. Representative immunoblots (A) and densitometric quantification (B) are presented. N = 4.

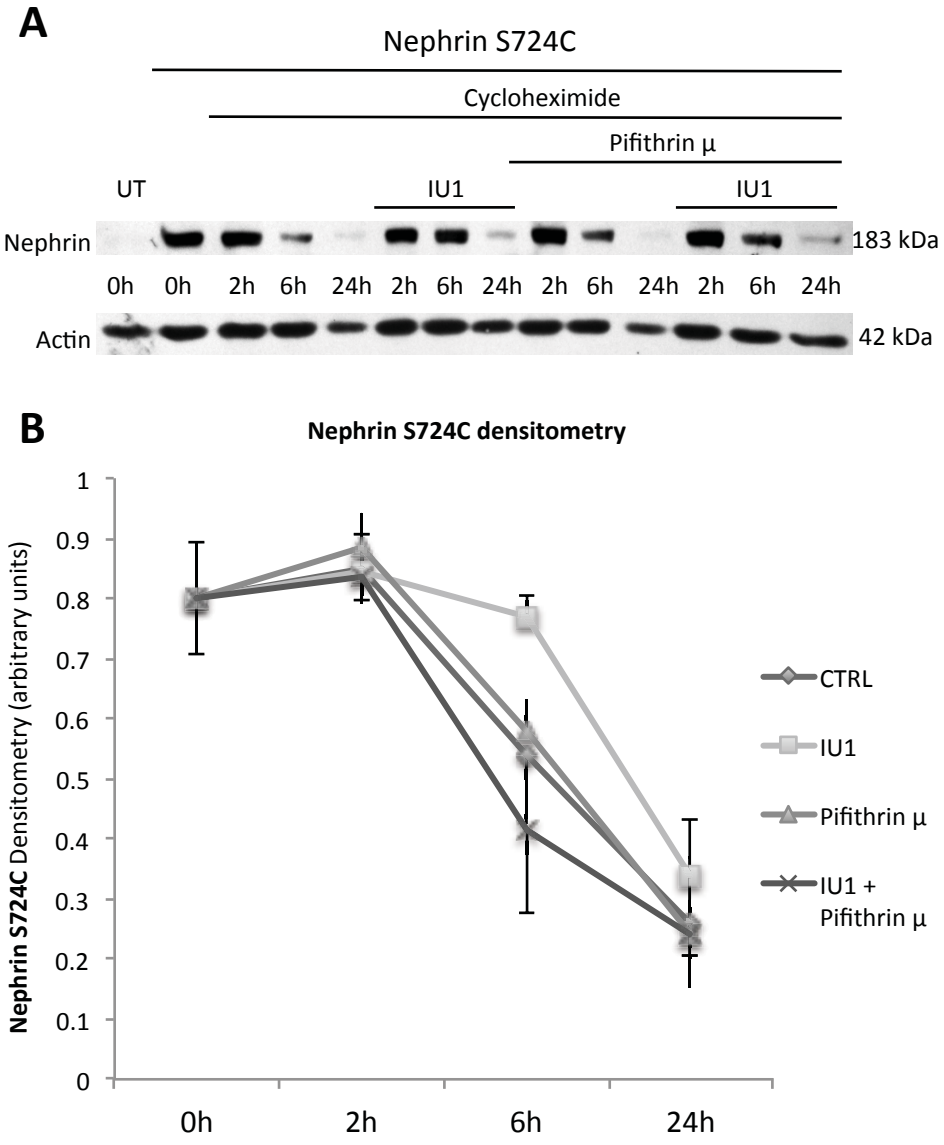


Fig. 9. Usp14 inhibitor, IU1, did not accelerate the degradation of nephrin mutant S724C in HEK 293T cells.

HEK 293T cells were transfected with human nephrin mutant S724C. After 48 h, transfected cells were treated with cycloheximide (to block protein synthesis), IU1 (50 μM), pifithrin μ (Hsp70 inhibitor; 5 μM), or with both IU1 and pifithrin μ. Each of these conditions was tested for at either 2, 6 or 24 h, with untransfected and untreated (0 h) controls. Cell lysates were immunoblotted with antibodies to nephrin and actin. Representative immunoblots (A) and densitometric quantification (B) are presented. N = 4.

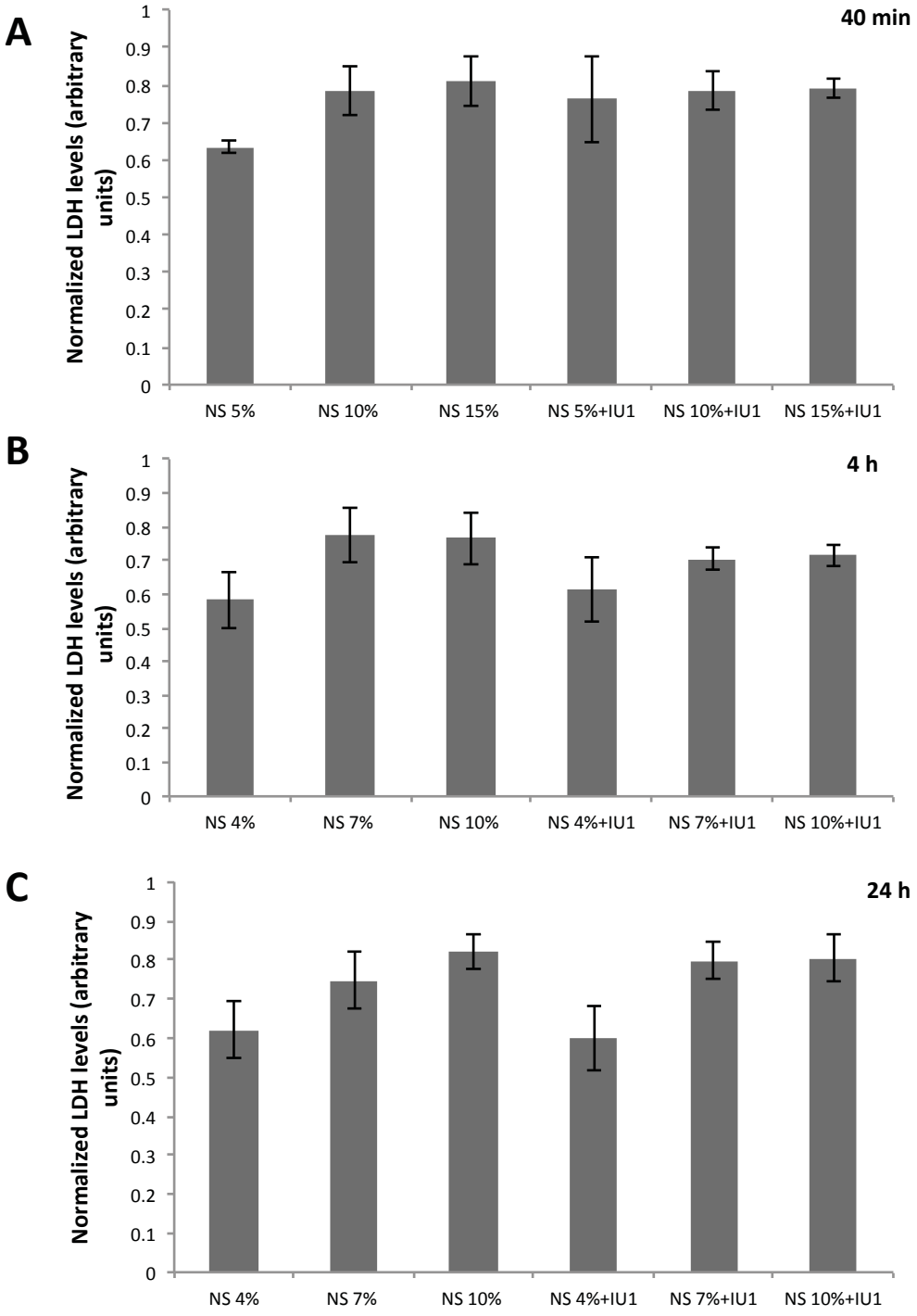

A
40 min
Normalized LDH levels (arbitrary units)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NS 5%
NS 10%
NS 15%
NS 5%+IU1
NS 10%+IU1
NS 15%+IU1
B
4 h
Normalized LDH levels (arbitrary units)
NS 4%
NS 7%
NS 10%
NS 4%+IU1
NS 7%+IU1
NS 10%+IU1
C
24 h
Normalized LDH levels (arbitrary units)
NS 4%
NS 7%
NS 10%
NS 4%+IU1
NS 7%+IU1
NS 10%+IU1

Fig. 10. Usp14 inhibitor, IU1, did not affect complement-induced cytotoxicity in glomerular epithelial cells.

GEC were pretreated with IU1 (50 μM) or medium alone for 30 min. C5b-9 assembly was induced by incubating cells with anti-GEC antiserum for 40 min and then adding normal serum (at the concentrations indicated) with or without IU1 (50 μM), or heat-inactivated serum (10-15%) in controls for 40 min (A), 4 h (B) or 24 h (C). Cytotoxicity was monitored by LDH release, and was normalized to 15% (A and B) or 10% (C) HIS. (A) N=3. (B) N=4. (C) N=5.

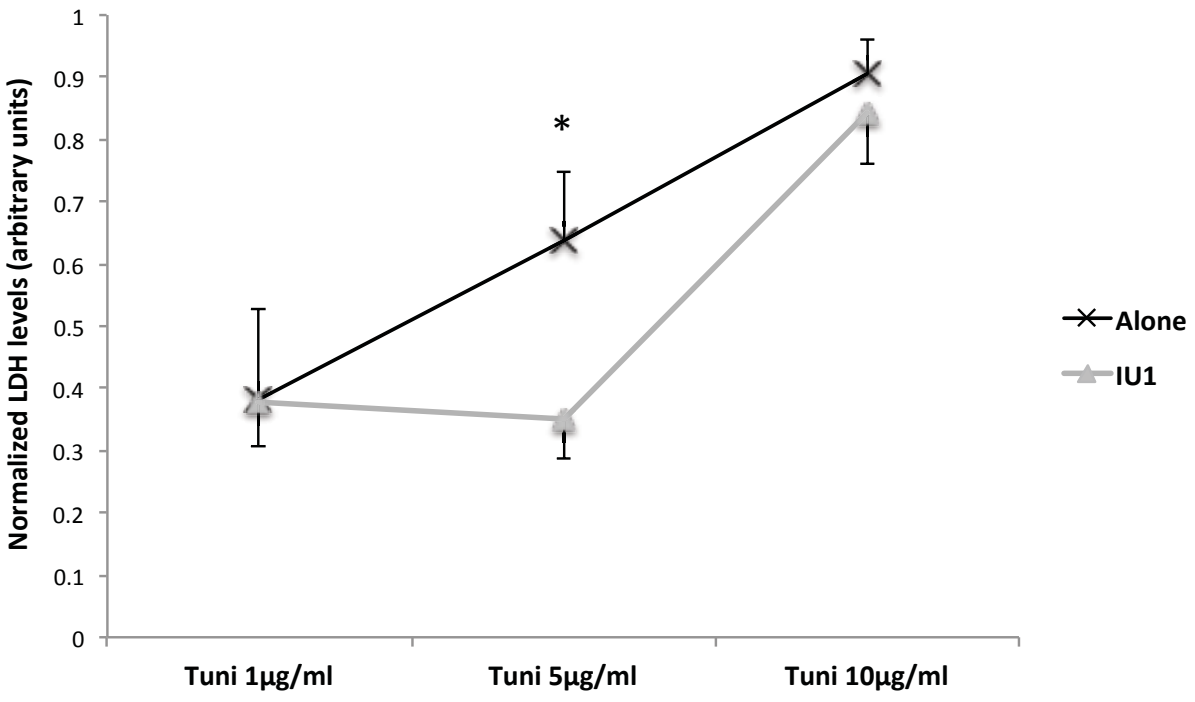


Fig. 11. Usp14 inhibitor, IU1, in GEC attenuated tunicamycin-mediated cytotoxicity. GEC were pretreated with IU1 (50 µM) or medium alone for 30 min. Then, cells were incubated with tunicamycin at different concentrations (1 µg/µl, 5 µg/µl or 10 µg/µl) with or without IU1 (50 µM) for 24 h. Cytotoxicity was monitored by LDH release, and was normalized to either untreated or IU1 alone. *$p<0.05$ (Tuni 5 µg/µl vs. Tuni 5 µg/µl +IU1), N=5.

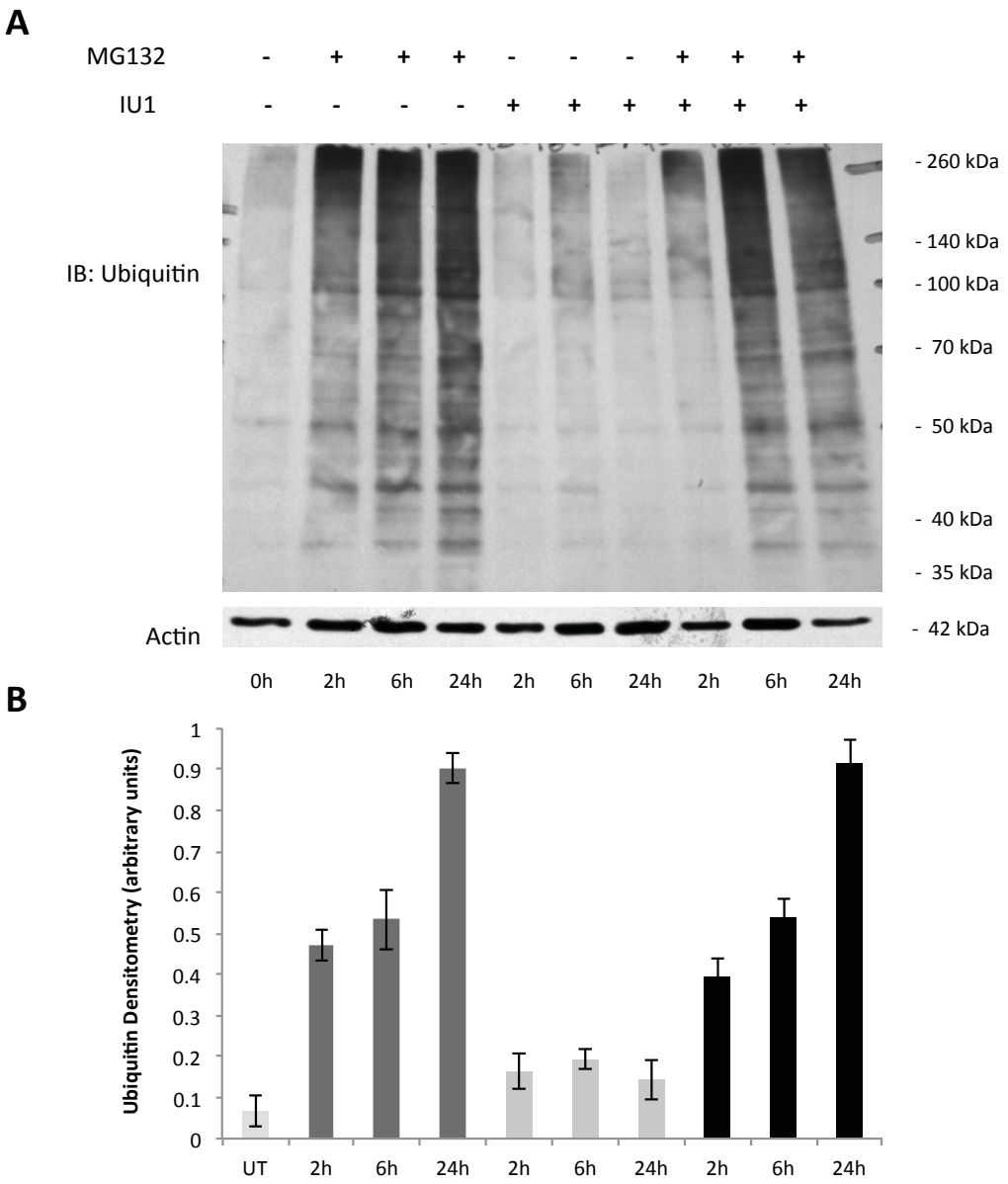


Fig. 12. Usp14 inhibitor, IU1, did not modify global ubiquitination of proteins significantly.

GEC were treated with either the proteasome inhibitor MG132 (25-50 μM) alone, IU1 (50 μM) alone or both MG132 plus IU1. (A) Cell lysates were immunoblotted with antibodies to ubiquitin and actin. Representative immunoblot is presented. (B) Densitometric quantification of ubiquitin is presented. N=7.

Chapter 5: Discussion

Targeting the UPS in the treatment of diseases, particularly those involving protein misfolding, is a relatively new field of experimentation, but a seemingly obvious one. The present study demonstrates that Usp14 (a proteasomal-associated DUB) controls proteasomal degradation of some, but not all, misfolded proteins. Inhibition of Usp14 appears to be useful in alleviating the proteotoxic effects of specific misfolded proteins, and is effective in reducing cytotoxicity in the context of global protein misfolding during certain types of cell injury. Most proteins, both intra- and extracellular, are continuously turned over - broken down to their constituent amino acids, which are recycled for the translation of new proteins. Additionally, the UPS carries out a significant portion of the protein degradation in a cell. Proteasome inhibitors were initially researched to block the excessive breakdown of muscle proteins in patients with cachexia. It was observed that blocking this pathway, or more specifically, the proteasome, leads to cell death by activation of apoptosis. Inhibiting the proteasome has been a successful targeting mechanism for treatment of certain cancers, since cancer cells have aberrant signaling pathways rendering them prone to apoptosis. Since then, research into targeting the proteasome for treatment has taken off in multiple fields of medicine [61, 62].

This work opened doors for researchers to target other levels of the UPS, specifically at earlier steps of a protein substrate's life, and not simply at the last stage of proteasomal degradation. The UPS is implicated in many diseases, specifically those involving excessive protein breakdown, which in many cases relates to the kidney [61]. Therefore, targeting this crucial pathway in research, and eventually in treatment, is a logical step for research in nephrology.

Some of the first studies of ER stress in glomerular diseases involved experimental models of membranous nephropathy, and increasing evidence is confirming the link of ER stress with proteinuric kidney disease [19]. Studies in GEC showed that disruption of the ER membrane after complement activation led to disruption of protein folding in the ER. The ER responds to this stress by induction of ERAD and UPR, and notable changes occur in UPS

activity in kidney disease [19, 27]. Preconditioning of rats with subnephritogenic doses of compounds that activate the UPR resulted in less severe proteinuria in animals with experimental membranous nephropathy, compared to non-preconditioned animals. This implied that an enhancement of the UPR before an insult reduces injury [19]. Since the main causes of glomerulonephritis are still largely unknown and pretreating potential kidney patients is impractical for obvious reasons, targeting treatment at an early phase after diagnosis is the next best option.

An earlier study showed that the deubiquitinating enzyme, Usp14, delayed the degradation of several proteins, some of which are implicated in neurodegenerative disease. A small molecule inhibitor of Usp14 was also identified, which was employed to enhance proteasomal activity [47]. The aim of our studies was directed towards understanding the role of Usp14 in the UPS in the pathogenesis of glomerulonephritis. Overexpression of Usp14 in GEC revealed a delay in UPS functioning, as monitored by UPS reporter, GFP^u (Fig. 2). This provided support for our hypothesis that Usp14 could be a target for inhibition in kidney disease.

Complement C5b-9 induces sublethal glomerular injury and proteinuria. Our lab previously showed that C5b-9 also enhances the UPS, by looking at cytosolic GFP^u degradation. It was speculated that this result could be in part due to the observation that ubiquitination also increased upon complement stimulation, while 20S proteasome activity decreased slightly [12]. However, this does not rule out that the proteasome could be working more efficiently despite its activity being slightly reduced. This is supported by the concept that monitoring UPS function using GFP^u (a specialized reporter) takes into consideration the functioning of the entire UPS [49, 50]. Consistent with the results of the previous study, we showed that activation of complement C5b-9 did indeed enhance the UPS, as demonstrated by the accelerated degradation of GFP^u (Fig. 3). We then employed the small-molecule inhibitor of Usp14, IU1, in GEC stimulated with complement. IU1 treatment significantly enhanced the degradation of GFP^u on top of the already enhanced degradation that was carried out by complement stimulation alone (Fig. 3). These results indicate that in GEC, UPS functionality increases in pathological states, compared to normal physiological states.

The enhancement of UPS by blocking Usp14 after complement stimulation indicates that Usp14 plays a significant role in delaying proteasomal degradation in a stressed state.

While GFP^u assesses protein turnover in the cytosol and nuclear compartments, it has some limitations [49]. The ER compartment is the site of important modifications of secretory proteins, and this takes place with the use of distinct ER chaperones [20]. Our lab also previously examined ERAD functioning in GEC after induction of ER stress. Degradation of the ERAD reporter, CD3δ-YFP, was delayed upon complement activation, or when N-glycosylation was inhibited by addition of tunicamycin [12]. This implies that despite enhanced UPS function, complement impaired ERAD, which could be due to an excess of misfolded proteins accumulating in the ER. These misfolded proteins may be competing for the ER resident chaperones, and for retrotranslocation out of the ER and into the cytosol for proteasomal degradation. We found that Usp14 overexpression tended to delay the degradation of CD3δ-YFP (Fig. 4). Additionally, we noticed that the activation of complement did not change the levels of CD3δ-YFP substantially, and treatment with IU1 only tended to enhance degradation of CD3δ-YFP after complement stimulation (Fig. 5). These results support previous observations, that despite the enhancement of the UPS in the cytosol, there is a delay in the ER compartment, which does not allow ERAD substrates to accelerate their degradation in the same manner as their cytosolic counterparts. A recent discovery of an ERQC compartment, which assembles during ERAD activation, has suggested that misfolded ER proteins get localized to this ERQC compartment prior to retrotranslocation via purported translocon, Sec61. It was shown that ubiquitination and proteasomal degradation machinery assembled near this ERQC during ERAD [63]. Misfolded proteins in the ER therefore may not have the same free access to the UPS machinery that cytosolic proteins do and are retained in the ER, which may explain why UPS reporter GFP^u degradation was enhanced with Usp14 inhibition, while ERAD reporter CD3δ-YFP degradation was not. Investigating the mechanism and rate by which substrates are retrotranslocated out of the ER during physiological situations compared with pathological situations may provide additional insight into our results.

The transmembrane glycoprotein nephrin is the principal component of filtration slit

diaphragms located in podocyte foot processes. Nephrin requires proper folding and modification in the ER, and only correctly folded proteins are transported to the Golgi apparatus. Nephrin reaches the cytosolic membrane to form a zipper-like structure and filtration channels to prevent large molecules from passing from the plasma into the urine [13]. Our lab previously studied several nephrin missense mutations, which show impaired glycosylation, retention in the ER, and prevention of export by certain mutants to the plasma membrane. Expression of these mutants resulted in the activation of the UPR [56]. One of these mutants, I171N, showed retention in the ER, while another, S724C, was apparently exported to the plasma membrane; both were shown to be misfolded, targeted for ERAD and degraded by the proteasome [16, 56]. We examined nephrin mutants I171N and S724C as a physiologically relevant model of ERAD to support our CD3δ-YFP experiments. When Usp14 was overexpressed in 293T cells, co-transfected levels of I171N (Fig. 6) and S724C (Fig. 7) mutants were shown to be significantly higher 4 h after treatment with cycloheximide (to block protein synthesis). Degradation rates are difficult to assess 24h post-co-transfection because the overexpression of Usp14 might start to delay degradation before cycloheximide treatment is initiated, and for this reason we did not normalize the nephrin mutants at the 0 h time point. Nonetheless, Usp14 resulted in a higher level of nephrin mutant expression, implying that Usp14 delays degradation of ERAD substrates, as well as the previously mentioned UPS reporter. One reason why these experiments may have provided distinct differences in degradation compared with the CD3δ-YFP experiments, could be because 293T cells can be transfected to a greater efficiency with nephrin mutants, whereas transfection efficiency is low in GEC expressing CD3δ-YFP (results not shown).

In keeping with our previous theme, we addressed the effect of Usp14 inhibition on the degradation rates of the nephrin mutants I171N and S724C. Treating 293T cells with IU1 did not have any significant effect on the degradation rates of both nephrin mutants after the addition of cycloheximide. In addition, adding an inhibitor of cytosolic chaperone Hsp70, also failed to show any difference (Fig, 8 and 9). The question arises as to why overexpressing Usp14 resulted in a delay in the degradation of the nephrin mutants, yet inhibition of Usp14 with IU1 did not enhance degradation. This could perhaps be explained

by previous observations that both Usp14 and its yeast homologue (Ubp6) display both catalytic and non-catalytic methods to delay proteasomal functioning [46, 47]. A plausible explanation is that increased activity of Usp14 could be deubiquitinating UPS substrates early, resulting in rescue from degradation, while simultaneously inhibiting the proteasome through its non-catalytic activity; a two-pronged mechanism. In contrast, inhibition by IU1 only blocks the deubiquitinating activity of Usp14, while presumably not affecting the non-catalytic activity, which is therefore still capable of affecting a delay on the proteasome. Nonetheless, since I171N and S724C are both missense nephrin mutations that result in protein misfolding in humans, they can be used as effective "physiological" ERAD reporters that can accompany or substitute "designed" reporters, such as CD3δ-YFP.

After demonstrating the role of Usp14 on the degradation of proteasomal substrates, we examined whether or not targeting Usp14 might protect GEC from cytotoxicity. Complement induces ER stress in GEC [11], and our lab previously found that complement increased cytotoxicity in a dose-dependent fashion. Additionally, inhibition of the proteasome enhanced complement-mediated cytotoxicity in cells [12]. This implied that the activation of the UPS by complement had a cytoprotective effect in GEC. In contrast, upon inhibition of Usp14 with IU1, we did not observe any significant protection of GEC from complement-mediated cytotoxicity (Fig. 10). In vivo, podocyte injury does not typically involve podocyte death; rather, podocyte foot process effacement and proteinuria are the hallmarks of glomerular disease, and specifically membranous nephropathy [2]. By analogy to the observation that UPS inhibition exacerbates the cytotoxic effect of complement in cultured GEC, UPS inhibition exacerbates proteinuria in experimental membranous nephropathy in vivo. In future studies, it will be important to address the role of Usp14 inhibition in vivo.

Another limitation of the experiment on complement-mediated cytotoxicity is the complex nature of complement signaling. Although complement does induce ER stress, many other signaling mechanisms are activated, which can have a multitude of effects on cell function simultaneously. For this reason, we chose to assess the role of Usp14 in ER stress by using a more specific cell stressor, i.e. tunicamycin. This drug inhibits N-glycosylation of proteins

in the ER, causing protein misfolding, which results in ER stress directly and a delay in the degradation of ERAD substrates [12]. Inhibiting Usp14 with IU1 in GEC resulted in significantly less cytotoxicity at an intermediate dose of tunicamycin treatment (Fig. 11). This experiment is consistent with a previous study, which showed that IU1 reduced cytotoxicity in HEK293 cells when oxidative stress was induced. These previous results showed a similar dose-dependency as our experiment, in that the largest protection of cell viability occurred at intermediate doses of injury with the oxidizing agent [47]. The results supports a cytoprotective effect of Usp14 inhibition during certain types of cellular stresses, and furthermore, IU1 may be protective at intermediate levels of stress, but the protection is overwhelmed when the stress becomes too great, or redundant when the stress is only modest.

Experimental models of GEC injury *in vivo* (membranous nephropathy) were associated with increases in total protein ubiquitination [12]. Usp14 and UCHL5 (the name for Uch37 in mice) are two of the three proteasome-associated DUBs, which deubiquitinate proteasomal substrates. A recent study described a small molecule inhibitor of both Usp14 and UCHL5, which resulted in enhanced degradation of proteasomal substrates in cells. In addition, inhibiting both Usp14 and UCHL5 resulted in accumulation of polyubiquitinated proteins similar to when the 20S core particle of the proteasome was inhibited. Of note, inhibition of both Usp14 and UCHL5 resulted in a shift of polyubiquitinated proteins to higher molecular weights, implying substrates acquiring increasingly longer ubiquitin chains. Problems in ubiquitin turnover generally represent a delay in proteasomal functioning and it is thought there is a degree of redundancy between the two DUBs, in that they both delay the proteasome and can take over the roles of the other [60]. We observed that blocking Usp14 with the specific inhibitor, IU1, did not modify global ubiquitination, nor did it result in a shift of proteins to higher molecular weight polyubiquitinated substrates (Fig. 12). These results are consistent with a previous study, which showed that inhibiting either Usp14 or Uch37 using RNAi enhanced the UPS, but inhibition of both together resulted in an accumulation of cellular polyubiquitinated proteins. Thus, the two DUBs have overlapping function, but at least one is necessary for efficient protein degradation [38]. A plausible explanation is that the deubiquitination mechanism of Usp14

is crucially important for maintaining ubiquitin homeostasis, as seen by Usp14 loss-of-function mice that develop ataxia, which is prevented by expressing neuronal ubiquitin[64]. Taking into account the importance of the need to restore the pool of free-ubiquitin, targeting inhibition of Usp14 alone, while leaving Uch37/UCHL5 intact, is still a potential approach to the enhancement of proteasomal function.

In conclusion, the results of the current and past studies demonstrate the importance of the relationship between Usp14 and proteasomal functioning. We have demonstrated that Usp14 does delay the degradation of certain proteins, both cytosolic and transmembrane. Contrastingly, inhibiting the deubiquitinating (catalytic) activity of Usp14 enhances the degradation of cytosolic proteins, while being less effective with proteins that accumulate in the ER. Further studies are needed to discover the link between ERAD and UPS, specifically at the step of retrotranslocation out of the ER and how ER proteins reach the ubiquitin-proteasomal machinery. Inhibiting Usp14 could be a potential target for enhancement of UPS function. However, improving the UPS alone may not be sufficient to enhance degradation of proteins during ERAD, and may have to be coupled with an enhancement of retrotranslocation of proteins out of the ER. Targeting the UPS, and specifically Usp14, may help to provide a cytoprotective effect when done at the appropriate time and stage during the pathogenesis of glomerulonephritis. Lastly, the mysterious mechanism of non-catalytic delay of the proteasome might be influencing experiments whereby we attempted to inhibit Usp14. Thus, further elucidation of this mechanism is crucial to address for future studies.

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