Endoplasmic reticulum stress induces autophagy in renal proximal tubular cells

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Abstract

Background. Autophagy, an intracellular self-degradation system conserved throughout eukaryotes, plays an important role in a variety of biological processes, including cell death, development, cancer, defence against infection and neurodegeneration. However, little information about autophagy in renal tubular cells is available. We investigated the relationship of autophagy with endoplasmic reticulum (ER) stress in proximal tubular cells.

Methods. Immortalized rat proximal tubular cells were exposed to the classic ER stress inducers tunicamycin or brefeldin A. Autophagy was detected mainly by immunoblot analysis of LC3, a widely used marker of autophagy, and also by immunofluorescent cytochemistry of LC3 and electron microscopy. Biological significance of the phenomenon was studied using bafilomycin A1, an inhibitor of autophagosome degradation. Signal transduction pathways following ER stress were also investigated using inhibitors of the MAPK pathway.

Results. Both ER stress inducers significantly increased LC3-II as a marker of autophagy in immunoblot analysis. Immunocytochemistry of LC3 and electron microscopy also showed activation of autophagy by ER stress. Inhibition by bafilomycin A1 showed that autophagy following...
ER stress fulfilled its intrinsic function, namely degradation of cytoplasmic components. Further, use of the MEK 1/2 inhibitor U0126, which inhibits ER stress-induced autophagy induction and ERK activation, showed that ERK, a MAPK family member, was necessary to the induction of autophagy. **Conclusions.** For the first time, we demonstrate the induction of autophagy following ER stress in renal tubules, and clarify its mechanism. These findings serve as the foundation for further investigation into autophagy in renal diseases.

**Keywords:** autophagy; ERK; ER stress; LC3

### Introduction

Cell death plays a key role under a wide range of pathophysiological conditions. Historically, two fundamental forms of cell death have been recognized, apoptosis and necrosis: apoptosis is a tightly regulated, energy-dependent process in which cell death follows a programmed set of events, while necrosis refers to the sum of degenerative changes that follow any type of cell death [1]. Under certain conditions, however, another form of cell death is seen: often termed type II (nonapoptotic) cell death, its essential feature is autophagy [2].

Autophagy is the term used to describe an intracellular self-degradation system found ubiquitously in eukaryotes. Of the three types recognized to date, macroautophagy, microautophagy and chaperone-mediated autophagy [3,4], that which occurs in type II programmed cell death is macroautophagy. Since the initial identification of its molecular components in yeast, termed autophagy-related genes (Atgs), knowledge about macroautophagy has rapidly expanded [5]. In macroautophagy (hereafter referred to as autophagy unless otherwise specified), a double membrane-bound structure called autophagosome is formed that sequesters cytoplasm, including one or more organelles of any type [6]. The autophagosome then fuses with a lysosome, after which its contents and inner membrane are degraded and recycled. While nutrient starvation is one typical stimulus that induces autophagy [4], it is also involved in physiological processes, such as development, differentiation and remodelling [7]. Moreover, recent studies have reported a role for autophagy under a variety of pathophysiological conditions, including cancer [8–10], defence against infections [11,12], neurodegeneration [13,14] and heart failure [15,16].

The role of autophagy in cell death thus depends on the context in which it occurs: it either constitutes a stress adaptation aimed at preventing cell death or conversely contributes to cell death. With regard to the kidneys, however, despite the critical role of tubular cells in both normal function and acute kidney injury [17] and chronic kidney disease (CKD) [18], a role for autophagy in tubular injury remains obscure. This lack of attention contrasts strongly with the extensive focus given to apoptosis in these cells.

One marker of injury to renal tubular cells is endoplasmic reticulum (ER) stress. ER stress can result from a number of ER disturbances, including oxidative stress, hypoxia and glucose deprivation. These cause the accumulation of misfolded proteins, which in turn invokes a response conserved throughout eukaryotes called the unfolded protein response (UPR) [19,20]. The UPR induces the expression of genes to maintain the homeostasis of the ER [21], but when it is overwhelmed, cells suffer from injury. We previously demonstrated that renal tubular cells suffer from ER stress under pathological conditions [22].

Several recent studies have reported that ER stress induces autophagy in mammalian cancer cell lines and mouse embryonic fibroblasts [23–25]. Here, we investigated whether ER stress induces autophagy in renal tubular cells. We also investigated the signalling pathway by which ER stress causes autophagy.

### Subjects and methods

**Reagents and antibodies**

The ER stress inducers tunicamycin and brefeldin A were purchased from Sigma. Bafilomycin A1 was purchased from Wako (Osaka, Japan). Tunicamycin, brefeldin A and bafilomycin A1 were stored in methanol, ethanol and DMSO solutions, respectively, at −20 °C. The mitogen-activated protein kinase (MAPK) inhibitor U0126 (MAPK and extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 inhibitor), SP600125 [c-Jun NH2-terminal kinase (JNK) inhibitor] and SB203580 (p38 inhibitor) were purchased from Calbiochem (San Diego, CA, USA) and stored in DMSO solution at −20 °C. Immunoblot analysis was done using rabbit polyclonal primary antibodies against the microtubule-associated protein 1 light chain 3 (LC3) (MBL, Nagoya, Japan), C/EBP homologous protein-10 (CHOP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pan-actin (Sigma), phosphorylated ERK 1/2 and total ERK 1/2 (Cell Signaling Technology, Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat antibodies against rabbit IgG (H+L) (Bio-Rad Laboratories, Hercules, CA, USA) were used as secondary antibodies.

**Cell culture and treatments**

The immortalized rat proximal tubular cell (IRPTC) is a cultured cell line derived from proximal tubular cells of 4-week-old male Wistar rats that are immortalized by transformation with origin-defective SV40 DNA [26]. These cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 25 mM HEPES at pH 7.4, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.1 mM non-essential amino acids at 37 °C under a humidified atmosphere of 5% CO2 and 95% air. After incubation in fresh complete medium for 90 min, IRPTC confluent on 20°C. Immunoblot analysis was done using rabbit polyclonal primary antibodies against the microtubule-associated protein 1 light chain 3 (LC3) (MBL, Nagoya, Japan), C/EBP homologous protein-10 (CHOP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pan-actin (Sigma), phosphorylated ERK 1/2 and total ERK 1/2 (Cell Signaling Technology, Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat antibodies against rabbit IgG (H+L) (Bio-Rad Laboratories, Hercules, CA, USA) were used as secondary antibodies.

**Immunoblot analysis**

After removal of the medium, treated cells were washed three times with ice-cold phosphate-buffered saline (pH 7.4), scraped off with a lysis buffer containing 2% Nonidet P-40, 0.2% sodium dodecyl sulphate (SDS), 50 mM Tris buffer (pH 7.4), 150 mM sodium chloride and a protease inhibitor cocktail. Complete Mini (Roche Diagnostics, Mannheim, Germany), incubated on ice for 30 min, and then centrifuged at 18 000 g for 20 min at 4 °C. The supernatant was used as a whole cell protein lysate. Cell lysates were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 15% gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with Tris-buffered saline (pH 7.4) with 0.5% (v/v) Tween 20 containing 5% skim milk, probed with the primary antibody against the target protein and a secondary HRP-conjugated antibody in sequence, and developed with chemiluminescence reagents (ECL Plus, GE Healthcare, Buckinghamshire, UK). Equal loading and transfer of proteins among lanes were verified by immunoblot analysis detecting actin. Band
Electron microscopy
After the indicated treatment, cells on a 60-mm dish were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) at room temperature for 1 h, post-fixed in 1% OsO4 in a 0.1 M phosphate buffer (pH 7.4) at room temperature for 1 h, dehydrated through a graded series of ethanol solutions and then embedded with Epon 812-filled EM-embedding capsules (TAAB Laboratories Equipment Ltd, UK) by heating the block on a hotplate and detaching it with the cells from the dish. Ultrathin (60-nm) sections were collected on the grid, stained with uranyl acetate and lead citrate and examined under a Hitachi H-7500 transmission electron microscope at 80 kV.

Statistical analysis
All values are expressed as means ± SD. Data for two groups were analysed using a two-tailed Student’s t-test, and those for more than two groups were compared using ANOVA. Differences with P-values <0.05 were considered significant.

Results

Effect of ER stress on autophagy in proximal tubular cells

During autophagosome formation, LC3, or Atg8, is converted from the unconjugated form (LC3-I) to the phosphatidylethanolamine (PE)-conjugated form (LC3-II), which is then incorporated into an autophagosomal membrane [27]. Immunoblotting of LC3 results in two bands, LC3-I at 16 kD and LC3-II at 14 kD. Although LC3-II has a greater molecular weight owing to the addition of PE, it migrates faster in SDS–PAGE due to its hydrophobicity. The amount of LC3-II correlates with the number of autophagosomes and is generally used to monitor autophagy [28]. Because nutrient starvation in EBSS, a condition typically used to elicit autophagy, increased LC3-II in IRPTC (Figure 1A), the induction of autophagy in renal proximal tubular cells by ER stress was determined using LC3 immunoblotting.

Results showed that autophagy was activated in IRPTC subject to ER stress using a representative ER stress inducer, tunicamycin (TM), in a time (Figure 1B)- and dose (Figure 1C)-dependent manner, as shown by the significant increase in LC3-II. This induction of ER stress by TM was confirmed by an increase in the immunoblot of CHOP, a representative ER stress marker, which corresponded to the increase in LC3-II (Figure 1B and C). Similar results were obtained using a second representative ER stress inducer, brefeldin A (BA) (Figure 1D and E). These results indicate that ER stress induced autophagy in renal proximal tubular cells.

Morphological verification of induction of autophagy by ER stress

We also morphologically confirmed the induction of autophagy by ER stress using LC3 immunocytochemistry and electron microscopy. On comparison with control cells, immunofluorescence studies of nutrient-starved tubular cells showed many clear dots of LC3 as a sign of autophagy (Figure 2A and C). In contrast, only weak background immunofluorescence was seen on incubation without the first antibody (Figure 2B). A similar dot pattern was seen on exposure to either ER stress inducer, TM or BA (Figure 2D–F).

On electron microscopy, autophagosomes were more conspicuous in starved than in control cells (Figure 3A and B), and more autophagosomes were similarly observed in cells treated with TM (Figure 3C).
Fig. 2. Immunocytochemistry of LC3. (A–C) IRPTC were starved in EBSS for 90 min (Starv) (C) or non-treated for the control (Cont) (A). Many conspicuous dots of LC3 are observed in starved cells only. The signal in the negative control without incubation with the first antibody is extremely weak (B). (D–F) IRPTC were treated with vehicle (Cont) (D), TM (0.5 µg/ml) (E) or BA (0.5 µg/ml) (F) for 8 h. Intense dots were seen in cells stimulated with ER stress inducers. Magnification is ×400. The scale bar shows 20 µm.

Fig. 3. Electron microscopic analysis of autophagosomes. Transmission electron micrographs of control cells (A), cells starved in EBSS for 90 min (B) and those treated with 2 µg/ml of TM for 8 h (C). Consistent with the results of immunoblot analyses of LC3 in Figure 1 and immunofluorescence studies in Figure 2, both nutrient starvation (B) and ER stress (C) increased autophagosomes (arrows). Magnification is ×40 000. The scale bar shows 0.5 µm.
Taken together, these findings show that ER stress induced autophagy in renal proximal tubular cells.

**Increased formation of autophagosomes by ER stress**

The increase in LC3-II, or autophagosomes, can be interpreted in one of two ways, either as an increase in the formation of autophagosomes or as a decrease in their degradation [28]. The former indicates the bona fide activation of autophagy because degradation of cargo is its intrinsic function. To determine which was correct here, we used bafilomycin A1 (Baf), which inhibits autophagosome-lysosome fusion [29] and the degradation of LC3-II integrated in the inner membrane of autophagosomes. Here, an increase in LC3-II on ER stress with the simultaneous use of Baf would theoretically indicate the contribution of the stress to the increase in autophagosome formation, i.e. induction of autophagy.

We first tested the effect of Baf itself on the autophagy of IRPTC. Baf caused a dose-dependent increase in LC3-II (Figure 4A), indicating that LC3-II in autophagosomal inner membranes was degraded in lysosomes and that autophagy in IRPTC fulfilled its intrinsic function, the degradation of its contents. Because the effect of Baf plateaued at 10 nM (Figure 4A), we used this dose in subsequent experiments.

We then treated tubular cells with an ER stress inducer and Baf together. As shown in Figure 4B, both TM and BA significantly increased LC3-II even in the presence of Baf, indicating that the increase in LC3-II by ER stress was not due to the decreased degradation of autophagosomes, but to the augmented formation of autophagosomes and activated functional autophagy.

**Essential role of ERK in ER stress-induced autophagy in renal proximal tubular cells**

The signalling pathway through which ER stress induces autophagy is largely unknown. Here, we evaluated the effect of MAPKs on the induction of autophagy. Three types of MAPKs are known, ERK, p38 and JNK [30]. ERK is phosphorylated and activated by MEK 1/2. To investigate its role in autophagy by ER stress, we used the MEK 1/2 inhibitor U0126 to block the ERK pathway. U0126 markedly blocked induction of autophagy by TM and BA (Figure 5). We also assessed the phosphorylated, or activated, form of ERK. Treatment with TM and BA increased phosphorylated ERK, while U0126 almost completely blocked this activation (Figure 5), indicating that the activity of ERK is necessary for the induction of autophagy by ER stress. In contrast, neither the JNK inhibitor SP600125 nor p38 inhibitor SB203580 decreased this autophagy (data not shown), showing that it was not dependent on JNK or p38.

**Discussion**

Here, we showed that the classic ER stress inducers TM and BA elicited autophagy in a rat renal proximal tubular cell line. This effect was not due to a decrease in the degradation of autophagosomes, but to the increased synthesis of new autophagosomes. Autophagy thus fulfilled its intrinsic function, the degradation of the contents of autophagosomes. Moreover, we also showed that MAPKs had different effects on this phenomenon, indicating that the induction of autophagy in these renal tubular cells was dependent on the ERK pathway and not the p38 or JNK pathways.

The primary finding of this study is that ER stress induces autophagy in tubular cells. Before the molecular components involved in autophagy, such as Atgs, were identified, several studies had reported appreciable macroautophagy in renal tubular cells, particularly in proximal tubular cells, mainly using electron microscopy [31–34], with adenosine 3',5'-monophosphate, parathyroid hormone [32], and the anticancer agent vinblastine [33] shown to increase
any treatment [43]. Although GFP-LC3 transgenic mice showed substantial LC3-II in podocytes, and speculated that these cells may show relatively high levels of autophagy in the basal state [42]. Mizushima et al. observed autophagy in podocytes in GFP-LC3 transgenic mice without any treatment [43]. Although GFP-LC3 transgenic mice serve as a useful tool for analysis of autophagy in vivo, high background of auto-fluorescence in tubules hindered our using this methodology to investigate autophagy in tubular cells (personal observation and reference 43).

ER stress in renal tubular cells has been shown to play a critical role in acute kidney injury in humans and in an animal model of ischaemia-reperfusion injury [44,45]. Known inducers in these cells include proteinuria, which is not only a marker of CKD but also a mediator of CKD progression [22], and several nephrotoxic agents, including paracetamol [46], cisplatin, gentamicin [47], cyclosporine A [48] and heavy metals [49]. These findings both emphasize the pathogenic role of ER stress and warrant further investigation of the role of ER stress-induced autophagy in the pathophysiology of renal diseases.

Recent reports have demonstrated that ischaemia-reperfusion injury, cisplatin and cyclosporine A elicited autophagy in proximal tubular cells [50–52]. The known role of these stimuli in inducing ER stress may suggest that the activation of autophagy under these conditions is mediated by ER stress. We plan to further investigate the relationship between autophagy and ER stress in renal disease models in vivo, using immunoblotting of LC3 and drugs that modify autophagy, such as rapamycin and chloroquine.

Although immunoblot analysis of LC3 is one of the most widely used methods for detecting autophagy, the characteristic that LC3-II levels can be enhanced by both increase in autophagosome formation and decrease in degradation means that results should be interpreted with caution. Interpretation is facilitated by agents that inhibit degradation, such as lysosomal protease inhibitors and bafilomycin A1 [28]; in particular, bafilomycin A1 inhibits vacuolar H⁺-ATPase, which lowers pH in some organelles, and it is this effect on autophagosome pH that likely explains how it blocks autophagosome–lysosome fusion [29]. In our study, ER stress increased LC3-II with bafilomycin A1, indicating that ER stress increased the formation of autophagosomes. Moreover, the enhancement of LC3-II by bafilomycin A1 also indicates the degradation of LC3-II and the contents of autophagosomes. Overall, ER stress activates functional autophagy in renal proximal tubular cells.

ERK has been shown to be necessary to the induction of autophagy by nutrient starvation [53,54] or a neurotoxin [55]. To our knowledge, the present study is the first to show that ERK activity is necessary to the induction of autophagy by ER stress.

While some studies on autophagy by ER stress in mammalian cells identified IRE1/JNK as a signalling pathway [23,36], we saw no involvement of JNK in ER stress-induced autophagy in tubular cells in our study. These discrepancies may be due to the difference in the methods used to induce ER stress, or in the type of cell used.

Although several links between ER stress and oxidative stress have been identified, their mechanism has not been fully elucidated [19,56]. Here, given that autophagy can also be induced by reactive oxygen species [57,58], we also investigated whether oxidative stress mediates the induction of autophagy by ER stress using the antioxidants N-acetyl cysteine and ascorbic acid, but saw no effect for either (data not shown). This suggests that oxidative stress is not

![Fig. 5. Involvement of ERK in autophagy by ER stress. IRPTC were incubated for 4 h in media including vehicle (Cont), tunicamycin (TM) (0.5 µg/ml) or brefeldin A (BA) (0.5 µg/ml), with or without U0126 (10 µM), an inhibitor of the ERK pathway. The harvested proteins were analysed by immunoblotting for LC3, phospho-ERK (p-ERK) and total ERK. U0126 abolished the enhancement of LC3-II and p-ERK by TM and BA. A representative immunoblot is shown; the graph shows the ratios of band intensities of LC3-II to those of actin, standardized to the mean of the control (n = 4). *P < 0.05.

![LC3-I, LC3-II, p-ERK, t-ERK, Actin](image-url)
involved in the activation of autophagy by ER stress, at least in the experimental system used here.

In conclusion, this study demonstrates that ER stress elicits functional autophagy in renal proximal tubular cells via the ERK pathway. These findings serve as the foundation for further investigation into autophagy in renal diseases.

Acknowledgements. This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (19390228) to M.N. and (19590093) to R.I. We are grateful to Noboru Mizushima (Tokyo Medical and Dental University, Japan) for his comprehensive and detailed advice on the detection of autophagy.

Conflicts of interest statement. None declared.

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Differential proteomic analysis of cyclosporin A-induced toxicity in renal proximal tubule cells

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Abstract

Background. The use of cyclosporine A (CsA) as a potent immunosuppressant has been limited by its severe nephrotoxic effects. The mechanisms involved are haemodynamic but also related to direct toxic effects of CsA on proximal tubule epithelial cells. We focused on defining a proteomic profile in CsA-treated proximal tubule cells to distinguish the direct impact of CsA on these cells from overlapping haemodynamically mediated phenomena that occur in an in vivo system.

Methods. By means of high-throughput differential proteomic analysis and mass spectrometry techniques in CsA and vehicle-treated proximal tubule-derived cell lines of human and mouse origin, we determined proteins that change their expression in the presence of CsA.

Results. CsA-induced toxicity analyses revealed that 10 mM CsA for 24 h was the threshold condition to induce significant changes in cell viability and proteomic profile. We identified 38 differentially expressed proteins on CsA-treated mouse PCT3 and human HK-2 cells, related to protein metabolism, response to damage, cell organization

Received for publication: 27.10.08; Accepted in revised form: 20.4.09