mTOR contributes to ER stress and associated apoptosis in renal tubular cells

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1Department of Cellular Biology and Anatomy, Georgia Regents University and Charlie Norwood Veterans Affairs (VA) Medical Center, Augusta, Georgia; 2Department of Nephrology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China; 3Department of Biochemistry and Molecular Biology, Georgia Regents University and Charlie Norwood VA Medical Center, Augusta, Georgia; and 4Cancer Center, Georgia Regents University and Charlie Norwood VA Medical Center, Augusta, Georgia

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Dong G, Liu Y, Zhang L, Huang S, Ding H, Dong Z. mTOR contributes to ER stress and associated apoptosis in renal tubular cells. Am J Physiol Renal Physiol 308: F267–F274, 2015. First published November 26, 2014; doi:10.1152/ajprenal.00629.2014.—ER stress has been implicated in the pathogenesis of both acute and chronic kidney diseases. However, the molecular regulation of ER stress in kidney cells and tissues remains poorly understood. In this study, we examined tunicamycin-induced ER stress in renal proximal tubular cells (RPTC). Tunicamycin induced the phosphorylation and activation of PERK and eIF2α within 2 h in RPTC, which was followed by the induction of GRP78 and CHOP. Consistently, tunicamycin also induced apoptosis in RPTC. Interestingly, mTOR was activated rapidly during tunicamycin treatment, as indicated by phosphorylation of both mTOR and p70S6K. Inhibition of mTOR with rapamycin partially suppressed the phosphorylation of PERK and eIF2α and the induction of CHOP and GRP78 induction during tunicamycin treatment. Rapamycin also inhibited apoptosis during tunicamycin treatment and increased cell survival. Collectively, the results suggest that mTOR plays a regulatory role in ER stress, and inhibition of mTOR may have potential therapeutic effects in ER stress-related renal diseases.

endoplasmic reticulum stress; mammalian target of rapamycin; tunicamycin; apoptosis; renal tubular cells

PROTEINS ARE SYNTHESIZED OR ASSEMBLED BY RIBOSOMES that are localized mainly on endoplasmic reticulum (ER). Newly synthesized proteins are further subjected to posttranslational modification in ER and Golgi apparatus for proper folding into native configurations, which are required for their trafficking and function. A well-controlled protein assembly and folding machinery in ER is vital to the maintenance of cellular homeostasis and viability. Accordingly, in response to the accumulation of misfolded or unfolded proteins, the cell activates a set of specific signaling pathways to cope with the stress, which is called unfolded protein response (UPR) or ER stress response (10, 28).

To date, three canonical signaling pathways of ER stress have been described (10, 28). First, ER stress may lead to the phosphorylation and activation of inositol-requiring enzyme 1 (IRE1), a unique enzyme with both kinase and RNAse activity that is required for specific splicing of Xbp1 (25). Spliced Xbp1 encodes a transcription factor to induce chaperone proteins and UPR genes involved in ER-associated protein degradation (ERAD). Second, upon ER stress, activating transcription factor 6 (ATF6) translocates to the Golgi apparatus, where it is activated by proteolysis. Proteolysed ATF6 then translocates into the nucleus to transcriptionally induce UPR genes for ERAD. Finally, in the PKR-like endoplasmic reticulum kinase (PERK) pathway, ER stress leads to the phosphorylation and activation of PERK, which further phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to the blockade of global protein synthesis and a concomitant induction of ATF4. ATF4, as a transcription factor, further induces the expression of downstream target genes such as C/EBP homologous protein (CHOP), a proapoptotic protein (27). Therefore, following UPR, the cell mounts a rapid stress response to shut down protein synthesis, enhance protein folding, and promote protein degradation; however, when ER stress is severe and overwhelming, cell death may be initiated via the PERK-CHOP pathway (27).

ER stress has been implicated in the pathogenesis of kidney diseases (4, 5, 12). For example, diabetic nephropathy in mice is associated with the induction of ER stress-related genes such as glucose-regulated protein 78 (GRP78), ATF4, and CHOP; moreover, manipulation of ER stress pharmacologically or genetically results in correlated changes in disease progress (2, 26). In acute kidney injury, ER stress has been documented in experimental models of ischemia-reperfusion injury and nephrotoxicity (22–24). Despite these interesting observations, little is known about the regulation of ER stress in renal cells and tissues under these pathological conditions. In the present study, we examined tunicamycin-induced ER stress response in renal tubular cells. Interestingly, we found that mTOR is activated rapidly during ER stress in these cells and contributes to subsequent cell death, revealing a role of mTOR signaling in ER stress in renal systems.

MATERIALS AND METHODS

Materials. The rat kidney proximal tubular cell (RPTC) line was originally obtained from Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH) and maintained as described previously (8, 14, 16, 17). Rabbit polyclonal antibodies, including anti-PERK, anti-phospho (Thr980)-PERK, anti-phospho (Ser51)-eIF2α, anti-mTOR, anti-phospho (Ser2481)-mTOR, and anti-phospho (Thr189)-p70 S6 kinase, were purchased from Cell Signaling Technology (Beverly, MA); monoclonal mouse anti-GRP78 was from BD Transduction Laboratories (Lexington, KY); mouse monoclonal anti-CHOP was from Cell Signaling Technology; mouse monoclonal anti-β-actin antibody was from Sigma (St. Louis, MO); and all secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Tunicamycin was purchased from MP Biomedicals (Solon, OH). Rapamycin was purchased from Sigma. Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) for caspase assay were purchased from Enzyme Systems Products.
GRP78 is induced as an adaptive mechanism to quench the ER stress pathways in an inactive state. During ER stress, GRP78 is the key molecular chaperone for keeping the treatment but was induced thereafter in a time-dependent manner. GRP78 is induced as an adaptive mechanism to quench the stress response. Consistently, GRP78 induction was detected in RPTC during 8–24 h of tunicamycin treatment (Fig. 1A). Densitometric analysis of the immunoblots further verified the phosphorylation of PERK and eIF2α and the induction of CHOP and Grp78 by tunicamycin time dependently in RPTC (Fig. 1B).

**RESULTS**

**Tunicamycin-induced activation of PERK pathway in RPTC.** To study ER stress in renal tubular cells, we treated confluent RPTC with tunicamycin, which inhibits N-linked glycosylation, a posttranslational modification required for proper folding in many proteins. Cell lysate was collected before tunicamycin was added or at various time points after tunicamycin was added. Tunicamycin induced a rapid activation of the PERK pathway of UPR or ER stress response. As shown in Fig. 1A, PERK phosphorylation was detected at 2 h of tunicamycin treatment, increased thereafter, and reached remarkable levels at 8–24 h. Downstream of PERK, eIF2 was also phosphorylated at 2 h of tunicamycin treatment; however, in contrast to PERK, eIF2 phosphorylation did not further increase at later time points. CHOP was not induced at 2 h of tunicamycin treatment but was induced thereafter in a time-dependent manner. GRP78 is the key molecular chaperone for keeping the ER stress pathways in an inactive state. During ER stress, GRP78 is induced as an adaptive mechanism to quench the stress response. Consistently, GRP78 induction was detected in RPTC during 8–24 h of tunicamycin treatment (Fig. 1A). Densitometric analysis of the immunoblots further verified the phosphorylation of PERK and eIF2α and the induction of CHOP and Grp78 by tunicamycin time dependently in RPTC (Fig. 1B).

**Tunicamycin-induced apoptosis in RPTC.** Despite initially being an adaptive response, ER stress triggers cell death when the stress is severe and becomes overwhelming (27). In RPTC, apoptosis was noticed after 12–16 h of tunicamycin treatment. By 24 h, a large percentage of cells underwent apoptosis. As shown in Fig. 2A, these cells showed typical apoptotic morphology, including cellular shrinkage and blebbing. Consistently, Hoechst 33342 staining also revealed nuclear condensation and fragmentation in these cells. Cell counting indicated that 53% of these cells became apoptotic at 24 h of tunicamycin treatment (Fig. 2B). Further biochemical analysis demonstrated a remarkable increase in caspase activity in tunicamycin-induced activation of PERK pathway in RPTC cells

**Measurement of caspase activity.** Caspase activity in cell lysate was measured as described previously (3, 7, 8, 29) using DEVD-AFC, a fluorogenic peptide substrate. Briefly, cells were stained with 10 µg/ml Hoechst 33342 staining for 5 min and then examined by phase contrast and fluorescence microscopy. Apoptotic cells showed a characteristic morphology, including shrunken configuration and apoptotic blebs or bodies, and a condensed and fragmented nucleus. Four fields with ~200 cells/field were examined in each dish to estimate the percentage of apoptosis. Representative images were also recorded.

**Morphological examination of apoptosis.** Apoptotic cells were identified by their morphology, as described previously (3, 7, 8, 29). Briefly, cells were stained with 10 µg/ml Hoechst 33342 staining for 5 min and then examined by phase contrast and fluorescence microscopy. Apoptotic cells showed a characteristic morphology, including shrunken configuration and apoptotic blebs or bodies, and a condensed and fragmented nucleus. Four fields with ~200 cells/field were examined in each dish to estimate the percentage of apoptosis. Representative images were also recorded.

**Statistical analysis.** Statistical differences between two groups were determined by Student’s t-test and multiple groups by analysis of variance. Values are expressed as means ± SD. P < 0.05 was considered statistically significant.

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cin-treated cells (Fig. 2C). Together, these results indicate that tunicamycin induces typical ER stress in RPTC that is associated with cell death by apoptosis.

**mTOR activation during tunicamycin treatment of RPTC.** mTOR and related signaling are central to cell growth, proliferation, and survival in various tissues and organs, including kidneys (9, 20). In connection with the present study, an interplay between mTOR and ER stress response has recently been eluded (1). With this background, we hypothesized that mTOR may participate in the regulation of ER stress in renal cells and tissues. To test this possibility, we initially examined mTOR activation during tunicamycin treatment of RPTC. mTOR activation is commonly indicated by the phosphorylation of mTOR and its downstream substrate proteins, such as p70S6K. Tunicamycin treatment for 2 h induced a low yet detectable mTOR phosphorylation (Fig. 3A). At 4 h, mTOR phosphorylation reached a maximal level, and thereafter mTOR phosphorylation decreased somewhat, but it remained markedly higher than control (Fig. 3A). Consistently, higher p70S6K phosphorylation was detected at 8 h. Quantification of the immunoblots by densitometry further verified mTOR and p70S6K phosphorylation during tunicamycin treatment, which was indicative of mTOR activation during ER stress in RPTC (Fig. 3B).

**Suppression of tunicamycin-induced ER stress by rapamycin.** To determine the possible regulation of ER stress by mTOR, we tested the effect of rapamycin, a specific mTOR inhibitor that is efficacious at nanomolar concentrations. Rapamycin was added during tunicamycin treatment of RPTC. As shown in Fig. 4A, rapamycin was first verified for its inhibition of mTOR phosphorylation or activation at various time points of tunicamycin treatment. Further immunoblot analysis showed that...
rapamycin suppressed PERK phosphorylation during tunicamycin treatment. Rapamycin also suppressed eIF2α phosphorylation, although the effect was not as impressive as that of PERK. CHOP induction by tunicamycin was not notably attenuated by rapamycin at early time points (e.g., 4 h), but it was diminished at late time points of 8–24 h (Fig. 4A). Interestingly, the effect of rapamycin on GRP78 was marginal during tunicamycin treatment of RPTC. These observations were substantiated by quantifying the protein signals by densitometry (Fig. 4B), indicating that mTOR contributes to ER stress in RPTC.

Inhibition of tunicamycin-induced apoptosis by rapamycin in RPTC. ER stress induced by tunicamycin led to apoptosis in RPTC (Fig. 2). To determine whether mTOR is involved in apoptosis during the treatment, we examined the effect of rapamycin. As shown in Fig. 5A, middle, tunicamycin treatment for 24 h induced massive apoptosis, as indicated by cellular and nuclear condensation and fragmentation in many cells. The apoptosis was markedly suppressed by inclusion of 100 nm of rapamycin during tunicamycin treatment (Fig. 5A, right). Quantification by cell counting indicated that tunicamycin induced >50% apoptosis, which was suppressed to 26% by rapamycin (Fig. 5B). Consistently, tunicamycin induced a marked caspase activation, which was also suppressed by rapamycin (Fig. 5C). We further tested the dose dependence of the inhibitory effect of rapamycin on apoptosis. As shown in Fig. 5D, 20 nM rapamycin was as efficacious as 500 nM rapamycin in blocking tunicamycin-induced apoptosis in RPTC, further supporting a role of mTOR in apoptosis under the experimental condition.

Effect of rapamycin on cell survival following tunicamycin treatment in RPTC. To corroborate the effect of rapamycin on apoptosis, we further analyzed cell survival. To this end, after 24 h of tunicamycin treatment with or without rapamycin, RPTC were changed to fresh medium incubation for another 24 h. After these incubations, control cells without tunicamycin exposure became very confluent and developed clear tight junctions, with some “cell domes” formed as a result of fluid transport and accumulation under cell monolayer (Fig. 6A, left). In sharp contrast, most cells in the tunicamycin only-treated group died and detached from the dish (Fig. 6A, middle). Remarkably, a significant portion of cells were rescued by rapamycin during tunicamycin treatment, and as a result, these cells could repopulate the dish (Fig. 6A, right). MTT assay further confirmed the beneficial effect of rapamycin on long-term cell survival (Fig. 6B).

DISCUSSION

ER stress is a cellular response to the accumulation of misfolded or unfolded proteins. The initial response is to cope with the stress by inducing the expression of chaperones to facilitate protein folding, activating protein degradation, and reducing protein synthesis. However, cell death ensues when ER stress is severe and prolonged and overwhelms the initial adaptive response (10, 28). In line with this idea, our current study has demonstrated renal tubular cell apoptosis following severe ER stress-induced tunicamycin (Figs. 2 and 5). Importantly, using this model, we have demonstrated mTOR activation during ER stress and have provided further evidence for the involvement of mTOR in ER stress and the associated cell death (Figs. 3–6). As such, these observations support the cross-talk between ER stress and mTOR signaling.

Considering the complex signaling pathways revolving around ER stress and mTOR, it is surprising that only recently was their cross-talk suggested (1). For example, Kato et al. (18) showed that rapamycin could suppress ER stress-associated apoptosis during thapsigargin and tunicamycin treatment in NRK-52E. They further suggested that rapamycin selectively suppressed the IRE1 ER stress pathway without affecting PERK and ATF6 pathways. Consistent with that study, we demonstrated the inhibitory effect of rapamycin on ER stress-associated apoptosis in renal RPTC (Figs. 5 and 6). However, mechanistically we showed that rapamycin suppressed the PERK pathway, including the phosphorylation of PERK and eIF2α and induction of CHOP (Fig. 4). On the basis of the well-known proapoptotic effect of the PERK-CHOP pathway (10, 28), we postulate that mTOR may contribute to apoptosis in ER stress in part by regulating this pathway. The exact cause of the discrepancy between our and the previous studies is unclear, but it may be related to the differences in the experimental models tested. Although three main pathways of ER
stress (IRE, PERK, and ATF6) have been described, their sensitivities to mTOR regulation may vary depending on the cellular context and the severity of ER stress.

Rapamycin had remarkable, beneficial effects in ER-stressed RPTC. It not only suppressed apoptosis but also improved long-term cell survival (Figs. 5 and 6). Moreover, rapamycin was efficacious in promoting cell survival even at 20 nM, the lowest concentration tested in our study. As eluded above, by inhibiting mTOR, rapamycin may block ER stress signaling, including the IRE1-JNK and/or PERK-CHOP pathway. It remains elusive as to how mTOR regulates these pathways.

mTOR functions in two main protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are distinct in protein compositions and cellular functions. It is generally recognized that rapamycin has specificity toward mTORC1 (13). Accordingly, it is suggested that mTORC1 likely participates in the regulation of ER stress and associated apoptosis in our study. mTOR exerts its regulatory functions by phosphorylating specific protein substrates. Thus, it is plausible that mTOR may phosphorylate a key protein(s) in specific ER stress pathways (e.g., PERK) to downregulate its activity. Alternatively, mTOR may contribute to ER stress by inducing new protein synthesis and therefore triggering further protein overload in ER. This notion is supported by our observation that mTOR was rapidly activated during ER stress to result in the phosphorylation and activation of p70S6K (Fig. 2), a protein kinase of the ribosomal S6 subunit for protein synthesis. In this possibility, rapamycin, by blocking mTOR, shuts down new protein synthesis, and therefore, it alleviates protein overload in ER, resulting in the amelioration of ER stress. Apparently, the activation of mTOR and associated protein synthesis during ER stress seems paradoxical or counterintuitive, as the stressed cell is striving to reduce protein overload, especially misfolded proteins, in ER. It is intriguing why and how mTOR and new protein synthesis are activated under this condition.

Suppression of Tunicamycin-induced ER stress by Rapamycin

**Fig. 4.** Suppression of tunicamycin-induced ER stress by rapamycin. RPTC were treated with 1 μg/ml tunicamycin in the absence or presence of 100 nM rapamycin for 0–24 h as indicated. Cell lysate was collected for immunoblot analysis of p-mTOR, p-PERK, p-eIF2α, CHOP, GRP78, and actin (loading control). A: representative immunoblots. B: densitometric analysis of protein expression. The signal of each protein band was measured by densitometry and then divided by the actin signal in the same sample. Data are expressed as means ± SD (n = 3). To show the effect of rapamycin, the protein signals at each time point were compared. *P < 0.05.
Yet another pathway that may link mTOR and ER stress signaling networks is autophagy, which normally delivers cytoplasmic contents into lysosomes for degradation. mTOR is known to be the major negative regulator of autophagy, and as a result, rapamycin stimulates autophagy in a variety of cells and tissues, including kidneys (11, 15, 21). Notably, under pathological conditions, autophagy is cytoprotective, antiapoptotic, and pro-survival in kidney cells and tissues (11, 15, 21). These considerations suggest that the activation of mTOR in ER stress may suppress autophagy and limit its cytoprotective action. However, this possibility is not supported by the observation that ER stress is generally associated with autophagy induction and not inactivation (6). Nonetheless, further investigations need to delineate these possibilities for the mechanism whereby mTOR contributes to cell injury and death during ER stress to be understood.

ER stress has been implicated in a broad range of renal diseases involving different cell types in kidneys, such as podocytes, tubular cells, and interstitial cells (4, 5, 12). The demonstration of the detrimental role played by mTOR in ER stress in kidney cells suggests the potential of renoprotection by blocking mTOR in the disease conditions. This is particularly tantalizing since rapamycin, the specific inhibitor of mTOR, is in clinical use for immunosuppression and chemotherapy of certain cancer types. However, it is noteworthy that mTOR has an essential role in cell growth, proliferation,
metabolism, and other cell biological functions. Thus, blocking mTOR, for example by rapamycin, may adversely affect the regenerative or repair process that is vital for functional recovery of organs and tissues, including kidneys. In this regard, it has been reported that rapamycin delays kidney repair following renal ischemia-reperfusion injury (19). Despite this concern, rapamycin is a reversible inhibitor of mTOR, and therefore, it may be applied for a defined period of time for tissue protection and then washed off to facilitate the growth and proliferation of surviving cells for tissue repair.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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