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Autophagy is involved in endoplasmic reticulum stress induced cell death of rat hepatocytes

Junlin Zhang, Ph.D.¹, Michael W. Morris Jr., M.D.¹, Wanda A. Dorsett-Martin, D.V.M.¹, Luke C. Drake, B.S.², and Christopher D. Anderson, M.D.¹

¹Department of Surgery, University of Mississippi Medical Center, Jackson, MS

²Mississippi College, Clinton, MS

Abstract

Background—Both endoplasmic reticulum (ER) stress and autophagy have been shown to display dual roles in cell survival in multiple cell lines. There is a reported but poorly understood link between ER stress, autophagy, and cell death. We hypothesized that autophagy plays a role in ER stress dependent cell death in rat hepatocytes.

Materials and Methods—Primary hepatocytes isolated from both lean and obese male Zucker rats were cultured and treated with tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), and wortmannin (WM) for 12 hours. The ER stress associated genes glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) were examined via quantitative real time PCR. Immunostaining with microtubule-associated protein 1 light chain 3 (LC3) as well as electron microscopy were used to evaluate autophagy activity. Trypan blue exclusion was used to determine hepatocyte cell viability.

Results—In both lean and steatotic hepatocytes, we found that TM induced both CHOP and GRP78 mRNA expression. Cells with increased ER stress were undergoing increased autophagy and had a significant decrease in cell viability. Both TUDCA and 3MA treatments attenuated TM induced ER stress, autophagy, and cell death, while WM treatment reduced autophagy and cell death but without changing ER stress.

Conclusions—These data suggest that autophagy is a likely downstream mediator of ER stress induced cell death in rat hepatocytes. Further exploration of the link between autophagy and ER stress in hepatocyte injury will yield important information that may be leveraged for treatment of liver injuries such as ischemia reperfusion.

Keywords

ER stress; autophagy; hepatocyte; cell death; lean; steatotic

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Corresponding author: Christopher D. Anderson, MD, FACS, Associate Professor of Surgery and Medicine, Chief, Division of Transplant and Hepatobiliary Surgery, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, Phone: 601-815-3135, cdanderson@umc.edu.

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Introduction

Stresses, such as disruption of cellular energy level, the redox state, and calcium concentration, often result in accumulation of unfolded proteins in the endoplasmic reticulum (ER) --- a condition referred to as ER stress. To restore a stable cellular environment, a series of cellular responses occur, collectively named the unfolded protein response (UPR) (1). The UPR in mammalian cells is mediated through three transmembrane ER receptors, namely pancreatic endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1). In the resting state, the above three receptors are maintained inactive through binding with their chaperone, glucose-regulated protein 78 (GRP78). After exposure to unfolded proteins during ER stress, GRP78 dissociates from these receptors, resulting in activation of the UPR (2). The UPR promotes cell survival following exposure to mild ER stress (3). However, after severe ER stress exposure, cell death will occur (2). Hence, ER stress seems to play dual roles in cell fate depending on the intensity of ER stress.

Stresses to cells such as nutrient starvation and ER stress also trigger a self-digesting process called macroautophagy (referred to as autophagy hereafter). Autophagy is a conserved mechanism in eukaryotic cells that starts with the formation of autophagosome with cellular organelles enclosed within a double membrane, followed by fusion with lysosome resulting in degradation of the engulfed organelles. Subsequent release of the digested amino acid and peptide into cytoplasm allows them to be recycled to synthesize other functional proteins (4). The main function of autophagy is to clear unwanted components in the cytoplasm and is believed to be beneficial to cell survival. Nonetheless, a considerable body of literature shows that autophagy can also cause cell death under certain circumstances (5). Therefore, autophagy also seems to act as a double-edged sword in mediating cell fate.

We have recently shown the importance of ER stress in mediating liver ischemia and reperfusion injury, specifically in the steatotic liver (6). Other studies have investigated the relationship among ER stress, autophagy, and cell death using different models. In general, most studies suggest that increased ER stress will cause increased autophagy, which can promote either cell death (7, 8) or cell survival (9). This relationship also can vary depending on the cell lines (10) and the dosages of ER stress inducer (11). Others have also shown that ER stress and autophagy play important roles in various diseases (5, 12) especially liver diseases (13, 14). Therefore, understanding the crosstalk of ER stress, autophagy, and cell viability is important and can potentially improve treatments of these diseases. Based on our previous findings, we hypothesize that there is an important link between ER stress and autophagy in rat hepatocytes. In this study, we studied this link in both lean and steatotic primary hepatocytes. We show that increased ER stress results in increased autophagy and cell death, while decreased ER stress or autophagy improves cell survival in both lean and steatotic hepatocytes.

Methods and Materials

All procedures were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and complied with the Association for Assessment and Accreditation of Laboratory Animal Care International and National Institutes of Health guidelines.

1. Cell culture of primary hepatocytes

After one week acclimatization, 6 lean and 6 obese Zucker rats (Charles River, Wilmington, MA, 300 grams) underwent standard hepatectomy using isoflurane. The liver was placed on a filter membrane with a 20G angiocath cannulated in the portal vein. A Masterflex L/S microprocessor pump (Cole Parmer, Vernon Hills, IL) was connected to the angiocath. The liver was flushed with 100ml liver perfusion medium (Gibco, Grand Island, NY) followed by 100ml liver digest medium (Gibco, Grand Island, NY) for 45 minutes, both mediums contain Penicillin Streptomycin (1:100, Gibco, Grand Island, NY) and kept at 37°C. The liver was then disrupted, filtered through a 230um mesh followed by sterile cotton gauzes. The mixed liver suspension was washed twice with hepatocyte wash medium (Gibco, Grand Island, NY) at 4°C. Nonviable hepatocytes were removed by Percoll solution (Sigma, St Louis, MO). The hepatocyte cell pellet was washed and then suspended in complete hepatocyte culture medium (Lonza, Allendale, NJ). After calculating the live cell density by trypan blue exclusion, the hepatocytes were plated in different chambers at 1×10^5 cells/ml, and incubated at 37°C with 5% carbon dioxide for normal growth. The culture medium was changed after 6 hours, and the cells were allowed to grow until about 80% confluent.

2. Drug treatment

When the hepatocytes were about 80% confluent, the culture medium was removed and replaced with fresh culture medium containing either only 1ug/ml tunicamycin (TM, Sigma, St Louis, MO)---ER stress inducer, or 1ug/ml TM with 10mM tauroursodeoxycholic acid sodium salt (TUDCA, Calbiochem, Darmstadt, Germany)---ER stress inhibitor, or 1ug/ml TM with 5mM 3-methyladenine (3MA, Sigma, St Louis, MO)---autophagy inhibitor, or 1ug/ml TM with 1uM wortmannin (WM, Sigma, St Louis, MO)---autophagy inhibitor. Non-treated (NT) hepatocytes were used as control. After 12 hours, the hepatocytes were collected for analysis.

3. Quantitative real-time polymerase chain reaction (PCR)

The hepatocytes were harvested using 0.25% Trypsin-EDTA (Gibco, Grand Island, NY). Hepatocyte RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), and its concentration was measured by Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was acquired through the thermal cycler (Bio-Rad, Hercules, CA) using cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The mRNA levels were quantified by TaqMan probe-based quantitative real-time PCR (Bio-Rad, Hercules, CA). Primers used for PCR include housekeeping gene 18S, ER stress marker genes including GRP78---ER stress chaperone as well as C/EBP homologous protein (CHOP)---proapoptotic gene (Applied Biosystems, Foster City, CA). The measurement of each gene was repeated in triplicate. Increased CHOP and GRP78 mRNA levels represents

increased ER stress in the cells, while reduced CHOP and GRP78 mRNA levels represents decreased ER stress in the cells.

4. Immunohistochemistry and quantification

The hepatocytes grown on glass coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 15 minutes. After rinse, cells were first incubated in blocking buffer (1% BSA and 0.3M glycine in TBS) for 1 hour and then in rabbit anti- microtubule-associated protein 1 light chain 3 (LC3, 1:100, Cell Signaling, Danvers, MA) antibody overnight at 4°C. After rinse, the hepatocytes were incubated in Alexafluor 488 anti-rabbit IgG (1:100, Invitrogen, Grand Island, NY) at room temperature for 1 hour. Stained hepatocytes were then immersed in mounting medium with DAPI (Vectashield, Burlingame, CA) and coverslipped. Slides were examined using fluorescent microscope (Nikon E800) and representative photographs were taken using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). Twenty hepatocytes per treatment per animal were randomly selected and the numbers of LC3 puncta in these hepatocytes were counted, the average of the numbers was calculated to represent for the autophagy activity.

5. Electron microscopy

The hepatocytes were collected and fixed with 0.5% glutaraldehyde overnight and then post fixed with 1% OsO₄ for 1 hour. After dehydrating with series of ethanol, cells were processed with propylene oxide and finally embedded in epon. Pictures of representative hepatocytes were taken using transmission electron microscope.

6. Cell viability

After various drug treatments, the hepatocytes were harvested and suspended in hepatocyte culture medium. Standard trypan blue exclusion test was used to determine the percentage of live hepatocytes under the light microscope.

7. Statistical analysis

Two-Way ANOVA was used for omnibus test and Bonferroni test was chosen for the post hoc Test to determine statistical significance. P value less than 0.05 was considered significant. Data were expressed as mean±SD.

Results

1. Alteration of ER stress after different drug treatments

Compared to NT groups, TM treatment significantly increased CHOP mRNA levels in rat hepatocytes ($P<0.001$, 28.4 ± 12.8 in lean, 21.1 ± 5.9 in steatotic) (Fig. 1A). When compared to TM treatments, CHOP mRNA levels were significantly reduced in both TM+TUDCA groups ($P<0.001$, 4.4 ± 1.6 in lean, 7.1 ± 2.8 in steatotic) and TM+3MA groups ($P=0.011$, 10.9 ± 3.7 in lean, 16.1 ± 3.8 in steatotic) but remained unchanged in TM+WM groups ($P=1.000$, 28.5 ± 18.4 in lean, 20.3 ± 5.4 in steatotic). No significant differences were found between lean and steatotic cells in TM groups ($p=0.099$), TM+TUDCA groups ($p=0.56$), TM+3MA groups ($p=0.258$), and TM+WM groups ($p=0.093$).

Similar to the findings of CHOP mRNA levels, TM treatment also significantly increased GRP78 mRNA levels in rat hepatocytes ($P<0.001$, 16 ± 4.6 in lean, 9.3 ± 3 in steatotic) compared to NT groups (Fig. 1B). When compared to TM treatments, GRP78 mRNA levels were also significantly reduced in both TM+TUDCA groups ($P<0.001$, 4.6 ± 1.5 in lean, 5.3 ± 2.2 in steatotic) and TM+3MA groups ($P=0.002$, 7.6 ± 2.4 in lean, 8.2 ± 2.9 in steatotic) but did not show obvious change in TM+WM groups ($P=1.000$, 15.1 ± 3.8 in lean, 10.9 ± 3.7 in steatotic). Significant differences between lean and steatotic cells were detected in TM groups ($P<0.001$) and TM+WM groups ($p=0.022$) while no differences were found in TM+TUDCA groups ($p=0.688$) and TM+3MA groups ($p=0.704$).

2. Alteration of autophagy levels after different drug treatments

A baseline level of autophagy activity (measured by the number of LC3 puncta per hepatocyte) was detected in normal hepatocytes (3.8 ± 0.8 in lean; 5 ± 0.7 in steatotic) (Fig. 2). After TM treatment, autophagy levels were significantly increased ($P<0.001$, 24 ± 4.2 in lean, 26 ± 4.2 in steatotic) compared to NT groups. Compared to TM groups, autophagy levels were decreased in TM+TUDCA groups ($P<0.001$, 4.6 ± 0.9 in lean, 6.8 ± 1.3 in steatotic), TM+3MA groups ($P<0.001$, 6 ± 2.2 in lean, 8.4 ± 1.7 in steatotic), and TM+WM groups ($P<0.001$, 8.2 ± 1.3 in lean, 10.8 ± 0.8 in steatotic). No significant differences were found between lean and steatotic cells in NT groups ($p=0.396$), TM groups ($p=0.16$), TM+TUDCA groups ($p=0.124$), TM+3MA groups ($p=0.094$), and TM+WM groups ($p=0.07$). Representative photographs demonstrating autophagy profiles were shown in Figure 3 and Figure 4.

3. Alteration of cell viability after different drug treatments

After normalized to NT groups, the cell viability was significantly reduced in TM groups ($P<0.001$, 0.83 ± 0.11 in lean, 0.76 ± 0.14 in steatotic) compared to NT groups (Fig. 5). When compared to TM groups, cell viability was significantly increased in TM+TUDCA groups ($P=0.003$, 1.01 ± 0.03 in lean, 0.93 ± 0.06 in steatotic), TM+3MA groups ($P=0.036$, 0.99 ± 0.27 in lean, 0.88 ± 0.06 in steatotic), and TM+WM groups ($P=0.001$, 1.02 ± 0.06 in lean, 0.95 ± 0.07 in steatotic). No significant differences were found between lean and steatotic cells in TM groups ($p=0.261$), TM+TUDCA groups ($p=0.252$), TM+3MA groups ($p=0.076$), and TM+WM groups ($p=0.344$).

Discussion

The relationship of ER stress, autophagy, and cell death is complex due to the fact that both ER stress and autophagy have cytoprotective and cytotoxic effects. The ultimate effect of ER stress and autophagy on cell fate seems largely depend on the specific cellular environments such as the doses of the treatment and cell types. Here we reported that severe ER stress induces autophagy and cell death, while inhibition of ER stress and/or autophagy contributes to improved cell survival in both lean and steatotic rat hepatocytes. Together, these results suggest that autophagy is a downstream mediator of ER stress related cell death.

Mild ER stress activates all UPR sensors which promotes cell survival (15). In contrast, severe ER stress may cause cell death (2), and different molecular pathways have been

identified in this process (2, 16, 17). For example, it has been shown that excessive or persistent ER stress triggered cell death by activating CHOP and B-cell lymphoma 2 interacting mediator of cell death (Bim) protein levels (18). Elevated CHOP expression in turn resulted in down-regulation of B-cell lymphoma 2 (Bcl2) expression and enhanced oxidant injury (19). On the other hand, CHOP knockout mice showed alleviated cell death in response to ER stress suggesting a cytotoxic role of CHOP in mouse embryonic fibroblasts (20). Interestingly, it was further reported that the amino terminal region of CHOP serves an important modulatory role (i.e. regulation by P38) while the bZIP region of the molecule is responsible for inducing apoptosis (21). In addition, in mouse hepatocytes, palmitate-induced ER stress increased both CHOP and Jun N-terminal kinase (JNK) expression and resulted in increased cell death, while TUDCA treatment decreased CHOP and JNK expression and increased cell viability (22). Therefore, it is reasonable for us to deduce that enhanced CHOP mRNA level after TM-induced ER stress in rat hepatocytes at least partially accounts for the increased cell death noted in our current study.

In addition to these molecular pathways, recently, autophagy has been shown to be involved in ER stress induced cell death in different human tumor cell lines (12). In the current study, we report that autophagy is also an important mediator in ER stress induced cell death in rat hepatocytes. Nonetheless, the mechanisms of ER stress induced autophagy and cell death remain unclear. Several major pathways have been identified (23). For example, it was reported that TM-induced ER stress enhances autophagy by activating the Beclin1/PI3KC3 pathway (24). TM-induced ER stress also down-regulates mammalian target of rapamycin complex 1 (mTORC1), which results in increased autophagy in cultured mouse embryonic fibroblasts (8). Down-regulation of mTORC1 after TM-induced ER stress was also reported to be associated with increased expression of CHOP as well as regulated in development and DNA damage responses 1 (REDD1)---an inhibitor of mTORC1 (25). Similar findings were recently reported in mouse hepatocytes (26). In addition, as the downstream target of CHOP, Tribbles homologue 3 (TRB3) was also reported to inhibit mTORC1, which in turn induce autophagy after ER stress (7). Further studies are needed to determine whether these pathways are involved in our animal model. As for the relationship of autophagy and cell viability, many studies have reported that autophagy is primarily a pro-survival rather than a pro-death mechanism (27, 28). Although some studies argue that excessive autophagy might promote cell death and the intense degranulation may be the turning point that commits damaged cells to death process rather than cell protective process (29), the molecular mechanisms of autophagy induced cell death remain poorly understood.

Interestingly, we noticed that the phosphatidylinositol 3-kinase inhibitor 3MA decreased TM-induced GRP78 and CHOP mRNA levels in hepatocytes while WM failed to show such effect. This finding is supported by a recent report showing that 3MA decreased GRP78 and CHOP protein levels after TM treatment in human hepatocellular carcinoma cells (30). Another study reported that 3MA could decrease CHOP but not GRP78 protein levels, and that WM had no effect on CHOP and GRP78 protein levels in human cell lines (24). Taken together, it seems that WM inhibits autophagy but not ER stress, in contrast, 3MA seems to inhibit both pathways. Since both 3MA and WM could reduce autophagy and cell death after TM treatment, it suggests that autophagy is an important downstream mediator of ER stress-induced cell death in both lean and steatotic hepatocytes.

In summary, our results show that increased ER stress results in both enhanced autophagy and cell death in lean and steatotic hepatocytes. Decreasing ER stress and/or autophagy is protective to these cells. These data show that autophagy is an important mediator of ER stress-induced cell death in rat hepatocytes. These findings offer new insights into the mechanisms of human liver injuries such as hepatitis and ischemia reperfusion in which ER stress is known to play a large role. For example, after rat liver transplantation---a process of cold ischemia warm reperfusion to the donor liver, both ER stress and hepatocyte cell death were increased in lean and steatotic donor livers, while ER stress inhibitor TUDCA treatment greatly decreased ER stress and cell death (6). Other studies reported that autophagy activity and hepatocyte cell death were also enhanced in the donor livers after rat liver transplantation, and treatment with autophagy inhibitor WM reduced both liver damage and the mortality rate of recipient rats (31, 32). Therefore, targeting ER stress and/or autophagy seems to be a promising therapeutic avenue which deserves further investigation.

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Figure 1.

ER stress levels in lean and steatotic hepatocytes after drug treatments. A) CHOP mRNA levels were significantly increased in TM groups compared to NT groups, and CHOP mRNA levels were decreased in TM+TUDCA and TM+3MA but not TM+WM groups compared to TM groups. B) Similar to the change patterns of CHOP mRNA, GRP78 mRNA levels were also significantly increased in TM groups compared to NT groups, and GRP78 mRNA levels were decreased in TM+TUDCA and TM+3MA but not TM+WM groups compared to TM groups. Asterisk represents $P < 0.05$. Two-way ANOVA and post-hoc Bonferroni test were used. Non-treatment (NT), tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), wortmannin (WM).

Figure 2.

Autophagy activity in lean and steatotic hepatocytes after drug treatments. The number of LC3 puncta in hepatocytes was significantly increased in TM groups compared to NT groups, and significantly decreased in TM+TUDCA, TM+3MA, and TM+WM groups compared to TM groups. Asterisk represents $P < 0.05$. Two-way ANOVA and post-hoc Bonferroni test were used. Non-treatment (NT), tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), wortmannin (WM).

Figure 3.

Representative photographs showing LC3 immunoreactivity in lean and steatotic hepatocytes. In both lean (A1–A5) and steatotic (B1–B5) hepatocytes, note the increased number of LC3 puncta (green coarse dots, marker of autophagy) in TM treated cells (A2, B2) compared to NT groups (A1, B1), also note the decreased number of LC3 puncta in TM +TUDCA groups (A3, B3), TM+3MA groups (A4, B4), and TM+WM groups (A5, B5) compared to TM groups (A2, B2). Non-treatment (NT), tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), wortmannin (WM). Nuclei stained with DAPI (blue color). Scale bar is 5 μ m.

Figure 4.

Representative EM photographs demonstrating autophagy activity in lean and steatotic hepatocytes. In both lean (A1–A5) and steatotic (B1–B5) hepatocytes, autophagosomes (pointed by red arrows) were often found in TM treated cells (A2, B2), in contrast, only a few autophagosomes were identified in NT groups (A1, B1), TM+TUDCA groups (A3, B3), TM+3MA groups (A4, B4), and TM+WM (A5, B5). Non-treatment (NT), tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), wortmannin (WM), lipid (L). Scale bar is 1μm.

Figure 5.

Cell viability in lean and steatotic hepatocytes using trypan blue exclusion method. In both lean and steatotic groups, cell viability was significantly lower in TM groups compared to NT groups, however, cell viability was significantly higher in TM+TUDCA, TM+3MA, and TM+WM groups than TM groups. Asterisk represents $P < 0.05$. Two-way ANOVA and post-hoc Bonferroni test were used. Non-treatment (NT), tunicamycin (TM), tauroursodeoxycholic acid (TUDCA), 3-methyladenine (3MA), wortmannin (WM).