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World Journal of Gastroenterology
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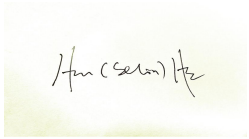
Date: 28th Nov, 2019

Dear Editor,

The article entitled "***Calcium-dependent calpain-2 activity promotes aberrant endoplasmic reticulum stress-related apoptosis in rat hepatocytes***" has been edited by *Medjaden* and its language quality is now considered to have reached Grade A.

Yours sincerely



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Name of Journal: *World Journal of Gastroenterology*

Manuscript Type: BASIC STUDY

Calcium-dependent calpain-2 activity promotes aberrant endoplasmic reticulum stress-related apoptosis in rat hepatocytes

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Authors' contributions

RX, BH and LZ performed the experiments. XH, SC, YC, YY and TY analyzed the data. RX and QY designed the study. RX wrote the manuscript. All authors approved the final manuscript.

Funding

This study was supported by the grants from the National Natural Science Foundation of China (no. 81560105), the Department of Science and Technology of Guizhou province [no. LH (2014) 7074].

Institutional review board statement

Not applicable.

Institutional animal care and use committee statement

Not applicable.

Conflict of interest statement

The authors declare no competing interests.

Data sharing statement

The datasets in the present study are available upon reasonable request.

ARRIVE guidelines statement:

The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines

ABSTRACT

BACKGROUND： Calpain-2 is Ca^{2+}-dependent cysteine protease. Studies have shown that aberrant calpain-2 activity is involved in apoptosis mediated by multiple triggers.

AIM: To investigate whether Ca^{2+}-dependent calpain-2 can modulate the aberrant endoplasmic reticulum (ER) stress-related apoptosis in rat hepatocyte BRL-3A cells.

METHODS: BRL-3A cells were treated with varying doses of dithiothreitol (DTT) and their viability and apoptosis were quantified by MTT and flow cytometry. The expression of ER stress- and apoptosis-related proteins was detected by Western blot. The activity of calpain protease was determined using a fluorescence substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC. The intracellular calcium contents and ER and calpain-2 co-location were characterized by fluorescent microscopy. The impact of calpain-2 silencing by specific siRNA on the caspse-12 activation and apoptosis of BRL-3A cells was quantified.

RESULTS: DTT exhibited dose-dependent cytotoxicity against BRL-3A cells and treatment with 2 mM DTT triggered BRL-3A cell apoptosis. DTT treatment significantly up-regulated 78kDa glucose-regulated protein (GRP78), activating transcription factor 4 (ATF4), C/EBP-homologous protein (CHOP) expression at protein level, and enhanced PRKR-like endoplasmic reticulum kinase (PERK) phosphorylation, caspase-12 and caspase-3 cleavages in BRL-3A cells in a trend of time-dependence. DTT treatment also significantly increased intracellular calcium contents, calpain-2 expression and activity in BRL-3A cells. Furthermore, immunofluorescence assay showed that calpain-2 could shift from cytoplasm to ER after DTT treatment, which provided new evidence for the involvement of calpain-2 in ER stress-mediated apoptosis. Moreover, calpain-2 silencing dramatically decreased calpain-2 expression and significantly mitigated the DTT-enhanced calpain-2 expression, caspse-12 cleavage and apoptosis in BRL-3A cells.

CONCLUSION: Such data indicated that Ca^{2+}-dependent calpain-2 activity promoted the

aberrant ER stress-related apoptosis of rat hepatocytes by activating caspase-12 in the ER.

Keywords: Calcium, Calpain-2, Caspase-12, Endoplasmic reticulum (ER) stress, Apoptosis, Hepatocyte

Core tip

Current information from experimental studies demonstrates that hepatocyte apoptosis is associated with many liver diseases. Calpain-2 has been implicated in the regulation of several apoptosis-related proteins through direct cleavage during apoptosis. However, the regulatory mechanisms by which calpain-2 regulates the ER stress-mediated hepatocytes apoptosis remain unclear. In this study, the effect of calpain-2 on ER stress-mediated hepatocyte apoptosis and underlying regulatory mechanisms were investigated. Our study indicates that calpain-2 is crucial for the aberrant ER stress-induced hepatocytes apoptosis and may provide a new therapeutic target for liver diseases.

INTRODUCTION

Hepatocyte apoptosis participates in the pathogenesis of many types of liver diseases [1-5]. However, the pathogenesis of hepatocyte apoptosis remains elusive. It is notable that aberrant endoplasmic reticulum (ER) stress can trigger hepatocyte apoptosis [6, 7]. While the ER is physiologically responsible for control protein proper folding and function, many factors, such as unfolded protein response (UPR), ER overload response (EOR) and others, can disturb ER function, leading to ER stress [8, 9]. Furthermore, aberrant ER stress can trigger cell apoptosis, particularly by activating caspase-12 [10-12]. However, how aberrant ER stress induces caspase-12 activation to trigger cell apoptosis has not been clarified in hepatocytes.

Calpain-2 is a Ca^{2+}-dependent cysteine protease that can cleave their protein substrates. Calpain-2 can regulate cell cycle, differentiation, and apoptosis [13]. Previous studies have revealed that calpains promote the ER stress-related apoptosis by activating caspase-12 [14-16]. Actually, our previous studies indicated that ER stress occurred in hepatocytes in a rat model of CCl_4-induced hepatic fibrosis, which were associated with increased calpain-2 and caspase-12 expression and hepatocyte apoptosis [17, 18]. However, it is unclear whether calpain-2 can modulate caspase-12 activation and ER stress-related apoptosis in hepatocytes.

This study explored the importance of calpain-2 in regulating caspase-12 activation and dithiothreitol (DTT)-induced ER stress-related apoptosis in hepatocytes. Our study indicated that calpain-2 activity was crucial for DTT-induced ER stress-related hepatocyte apoptosis by activating caspase-12 in vitro.

MATERIALS AND METHODS

Special reagents included Rat BRL-3A cells (Number: KCB92013YJ, Kunming Cell Bank of Chinese Academy of Sciences, China), Fetal bovine serum (FBS), dulbecco's modified Eagle's medium (DMEM, GIBCO, New York, USA), Acrylamide, bisacrylamide, ammonium

peroxydisulfate, glycine, Tri-hydroxymethyl aminomethane, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-H-tetrazolium bromide (MTT), Tween 20, dithiothreitol (DTT), dimethyl sulfoxide (DMSO, Genview, USA), *N*-succinyl-Leu-Leu-Val-Tyr-AMC (Sigma), antibodies against GRP78, PERK, ATF4, CHOP, and caspase-12 (Abcam, Cambridge, UK), antibodies against p-PERK (Affinity, USA), calpain-2 and β-actin (Cell Signaling Technologies, Massachusetts, USA), caspase-3 and secondary antibodies (Boster Biological Engineering, Wuhan, China), polyvinylidene difluoride (PVDF) membranes and enhanced chemiluminescence (ECL) kit (Millipore, Massachusetts, USA). The calpain-2 specific and control siRNAs, siRNA dilution buffer were produced by Santa Cruz Biotechnology (California, USA), and the Annexin V-FITC detection kit, ER-tracker Red and Fluo-3 AM were obtained from Beyotime Institute of Biotechnology, Nanjing, China.

MTT assay

We cultured Rat BRL-3A cells in 10% FBS DMEM at 37 ℃ in 5% CO_2 and quantified the impact of DTT on the BRL-3A cell viability by MTT. Briefly, BRL-3A cells (5×10^3 cells/well) were treated in triplicate with 0-10 mM of DTT for 24 h and the cell viability was examined using a microplate reader after adding MTT solution for 4 h.

Flow cytometry

We tested the cytotoxicity of DTT by flow cytometry using the Annexin V/propidium (PI) kit per the manufacturer's protocol. Briefly, BRL-3A cells (5×10^5 cells/flask) were treated in triplicate with vehicle or 2.0 mM DTT for varying time periods. The cells were stained with FITC-Annexin-V and PI and characterized by flow cytometry in a FACSCalibur™ (BD Biosciences).

Western Blotting

After treatment with DTT for varying time periods, BRL-3A cells were lyzed in RIPA buffer and centrifuged, followed by quantified the protein concentrations. The cell lysates (50 μg/lane) were separated by 10-12% SDS-PAGE and transferred onto PVDF membranes. After being blocked with 5% non-fat dry milk in TBST, the membranes were probed overnight at 4 ℃ with primary antibodies against GRP78 (1:1500), PERK (1:1500), p-PERK (1:1500), ATF4 (1:1500), CHOP (1:1500), calpain-2 (1:1000), caspase-12 (1:1000), cleaved capase-3 (1:500), and β-actin (1:1000). The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated second antibodies and visualized using ECL reagents. The signal intensity was measured using Bio-Rad imaging system (Bio-Rad, USA) and analyzed by Quantity one software (Bio-Rad, USA).

Confocal microscopy analysis of intracellular Ca^{2+} detection

The levels of intracellular Ca^{2+} in BRL-3A cells were imaged by confocal microscopy after staining with a Ca^{2+}-sensitive fluorescent dye, Fluo-3 AM. In brief, BRL-3A cells were treated in triplicate with 2.0 mM DTT for 0, 6, 12 and 24 h, and stained with Fluo-3 AM at 37℃ for 45 min. A half hour later, the fluorescent signals in individual wells of cells were examined under a confocal microscope (Olympus, FV1000, Japan).

Calpain activity assay

We measured cellular calpain activity [19]. In brief, individual cell lysates were reacted at 37 ℃ with *N*-succinyl-Leu-Leu-Val-Tyr-AMC for 1 h and the fluorescent signals were measured using a fluorescence plate reader.

Immunofluorescence microscopy

BRL-3A cells were cultured on coverslips overnight, and treated with 2.0 mM DTT for 0, 6, 12 and 24 h. The cells were stained with ER-tracker Red (ER fluorescent dye), fixed and permeabilized. Subsequently, we stained the cells with anti-calpain-2 and Alexa Fluor 488-conjugated secondary antibody. We photoimaged the fluorescent signals under a fluorescence microscope (Olympus, IX71, Japan).

Calpain-2 siRNA transfection

BRL-3A cells (5×10^5 cells/well) were grown in antibiotic-free medium overnight and transfected with control or calpain-2 specific siRNA for 48 h. The efficacy of calpain-2 silencing was determined by Western blotting. Subsequently, the different groups of cells were treated in triplicate with 2.0 mM DTT for 24 h and used for analysis of apoptosis, calpain-2 and cleaved caspase-12 expression.

Statistical analysis

Data are present as mean ± SD. We compared the different groups of data by one-way ANOVA and post hoc least significant difference (LSD) using SPSS13.0 software. Statistical significance was defined when a *P-value of* <0.05.

RESULTS

DTT exhibits cytotoxicity against BRL-3A cells in a dose-dependent manner.

To determine optimal dose of DTT, BRL-3A cells were treated with varying concentrations of DTT for 24 h and their viability was measured by MTT (Figure 1A). Apparently, DTT had dose-dependent cytotoxicity against BRL-3A cells and the LD50 value for BRL-3A cells was

about 4 mM in our experimental condition. Longitudinal analysis displayed that treatment with 2 mM DTT for 6 h significantly increased the percentages of apoptotic BRL-3A cells and treatment for a longer period did not significantly deteriorate BRL-3A cell apoptosis (Figure 1B and C). Hence, DTT exhibited dose-dependent cytotoxicity against BRL-3A cells by inducing their apoptosis in vitro.

DTT induces ER stress in BRL-3A cells

DTT can induce ER stress in many types of cells [20-22]. To understand the role of DTT in decreasing viability, BRL-3A cells were treated with 2.0 mM DTT for varying time periods and their ER stress-related proteins were quantified by Western blot (Figure 2). DTT treatment for 6 h up-regulated GRP78, ATF4 and CHOP expression and PERK phosphorylation in BRL-3A cells and treatment with DTT for a longer period further increased its effects. The up-regulatory effects of DTT on GRP78 expression and PERK phosphorylation appeared to be a trend of dose-dependence. Thus, DTT induced ER stress in BRL-3A cells in vitro.

DTT enhances caspase-12 and caspase-3 activation and calpain-2 activity in BRL-3A cells

Aberrant ER stress can promote sensitive cell apoptosis by inducing caspase-12 and caspase-3 activation and calpain-2 participates in the process of ER-stress-related apoptosis [15, 16, 23]. To understand the consequence of ER stress induced by DTT, the relative levels of caspase-3, and caspase-12 cleavage and calpain-2 activity were quantified. Treatment with 2 mM DTT significantly induced time-dependent caspase-12 cleavage and increased the levels of cleaved caspase-3 in BRL-3A cells (Figure 3A and B). Similarly, DTT treatment significantly up-regulated calpain-2 expression and enhanced calpain-2 activity in a trend of time-dependence in BRL-3A cells (Figure 3C and D). Given that calpain-2 activity is Ca^{2+}-dependent, we further quantified intracellular Ca^{2+} in each group of cells by microscopy. Following stained with Fluo-3

AM, we observed that DTT treatment time-dependently increased the contents of clustery Ca^{2+} in BRL-3A cells (Figure 4). To look for additional evidence to demonstrate the importance of calpain-2 activity, we stained the different groups of cells with fluorescent anti-calpain-2 and ER-tracker Red and observed the co-localization of red ER and green calpain-2 signals by fluorescent microscopy. As shown in Figure 5, there were obviously increased merged yellow signals in the DTT-treated cells, particularly in later time point while there was little merged signal in the DTT-untreated cells. Collectively, such data indicated that DTT-induced aberrant ER stress promoted of BRL-3A cell apoptosis by activating caspase-12 and increasing calpain-2 activity.

Calpain-2 silencing mitigates DTT-induced caspase-12 activation and apoptosis in BRL-3A cells

Finally, we tested whether calpain-2 silencing could modulate the DTT-induced caspase-12 activation and apoptosis of BRL-3A cells. We found that transfection with calpain-2 specific siRNA, but not the control, significantly decreased calpain-2 expression by about 85%, demonstrating the efficacy of calpain-2 silencing (Figure 5A). Calpain-2 silencing also significantly mitigated the DTT-up-regulated calpain-2 expression near 80% (Figure 5B). Although calpain-2 silencing did not alter the DTT-up-regulated caspase-12 expression, it did significant reduce the caspase-12 cleavage near 63% in BRL-3A cells, relative to that in the control cells (Figure 5C). More importantly, calpain-2 silencing dramatically decreased the percentages of DTT-induced apoptosis of BRL-3A cells by 50% (Figure 5D and E). Together, the significantly decreased caspase-12 activation and cells apoptosis indicated that calpain-2 activity was crucial for DTT-induced ER stress-related caspase-12 activation and apoptosis in BRL-3A cells.

DISCUSSION

Many factors can induce ER stress and they included chemical agents, such as tunicamycin, thapsigargin, and DTT [21, 24]. Aberrant ER stress can induce apoptosis of hepatocytes and participate in the pathogenesis of several types of liver diseases [25-27]. In this study, we found that DTT had strong cytotoxicity against rat hepatocyte BRL-3A cells and it cytotoxicity was dose-dependent. Furthermore, treatment with DTT induced ER stress in BRL-3A cells by significantly up-regulating GRP78, ATF4 and CHOP expression and PERK phosphorylation. More importantly, DTT treatment significantly increased the frequency of apoptotic BRL-3A cells, supporting the notion that aberrant ER stress promotes apoptosis of hepatocytes [28, 29]. Given that hepatocyte apoptosis participates in the pathogenesis of several types of liver diseases, inhibition of ER stress may be valuable for protection of hepatocytes from apoptosis.

The ER stress-related apoptosis is independent of mitochondria and death receptors, rather than is mediated by activating caspase-12 [30-32]. Activated caspase-12 can activate the downstream effector caspase-3, leading to apoptosis [33]. Actually, caspase-12 knock-out cells are resistant to ER stress-induced apoptosis [34]. We found that DTT treatment significantly induced caspase-12 and caspase-3 cleavages in BRL-3A cells. Such data indicated that the activated caspase-12 and caspase-3 contributed to the ER stress-related apoptosis of BRL-3A cells. Interestingly, we found that DTT treatment significantly increased intracellular calcium contents and calpain-2 expression and activity in BRL-3A cells. Furthermore, DTT treatment promoted the accumulation of calpain-2 in the ER of BRL-3A cells. These indicate that ER stress promotes calcium efflux from the ER and enhances calpain-2 activity and ER accumulation to cleave caspase-12 in hepatocytes. More importantly, calpain-2 silencing not only significantly mitigated the DTT-up-regulated calpain-2 expression and caspase-12 activation, but also decreased the DTT-triggered apoptosis of BRL-3A cells. Such data indicated that DTT-induced ER stress increased intracellular calcium contents and calpain-2 expression, leading to calpain-2 activation,

which cleaved caspase-12 to trigger apoptosis of BRL-3A cells. Such novel findings may provide new evidence to demonstrate that the Ca^{2+}-dependent calpain-2 activity is crucial for promoting the ER stress-related apoptosis in hepatocytes.

In conclusion, our data indicated that DTT exhibited dose-dependent cytotoxicity against rat hepatocytes and induced ER stress and apoptosis in BRL-3A cells. Evidently, DTT treatment significantly up-regulating GRP78, ATF4 and CHOP expression and PERK phosphorylation, increased intracellular calcium contents and calpain-2 activity and induced caspase-12 and caspase-3 activation in BRL-3A cells. Furthermore, calpain-2 silencing significantly mitigated the DTT-up-regulated calpain-2 activity and DTT-induced caspase-12 activation as well as apoptosis in BRL-3A cells. Such data suggest that ER stress may be new therapeutic targets and our findings may provide new evidence to demonstrate the importance of calcium-dependent calpain-2 in caspase-12 activation and ER stress-related apoptosis in hepatocytes.

Acknowledgements

We thank Hua Pei and Jinxingyi Wang for their technical advice for using flow cytometry and confocal microscope in the Basic Medical Science Research Center of Guizhou Medical University. We would like to thank Tengxiang Chen for his comments on this manuscript.

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Figure 1. DTT exhibits dose-dependent cytotoxicity against BRL-3A cells.

BRL-3A cells were treated in triplicate with, or without, the indicated doses of DTT for 24 h and their viability was examined by MTT (A). Subsequently, BRL-3A cells were treated with 2.0 mM DTT for varying periods and stained with FITC-Annexin-V and PI, followed by flow cytometry (B, C). Data are representative flow charts or expressed as the mean ± SD of each group from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$.

Figure 2. DTT induces ER stress in BRL-3A cells.

BRL-3A cells were treated in triplicate with, or without, 2.0 mM DTT for the indicated time periods, and the relative levels of GRP78, ATF4 and CHOP expression and PERK phosphorylation in each group of cells were quantified by Western blot. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments. (A) Relative GRP78 expression. (B) Relative PERK phosphorylation. (C) Relative ATF4 expression. (D) Relative CHOP expression. $^{*}P<0.05$, $^{**}P<0.01$.

Figure 3. DTT induces caspase-12 and caspase-3 activation and increases calpain-2 activity in BRL-3A cells.

After treatment with 2.0 mM DTT for varying periods, the relative levels of caspase-12, cleaved caspase-12 and cleaved caspase-3 as well as calpain-2 expression in each group of cells were quantified by Western blot. Furthermore, the activity of calpain-2 in each group of cells was measured. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments. (A) Caspase-12 activation. (B) Caspase-3 activation. (C) Calpain-2 expression. (D) Calpain-2 activity. $^{*}P<0.05$, $^{**}P<0.01$.

Figure 4. DTT increases the levels of intracellular Ca^{2+} in BRL-3A cells.

After treatment with 2.0 mM DTT for varying time periods, the cells were labeled by Fluo-3 AM

and the fluorescent signals were observed by microscopy, scale bars, 25 μm. Data are representative images from each group from three separate experiments.

Figure 5. DTT promotes the accumulation of calpain-2 in the ER of BRL-3A cells.

After treatment with 2.0 mM DTT for varying time periods, the cells were labeled with ER-tracker Red dye, fixed, perimeabilized, followed by stained with fluorescent-anti-calpain-2 (green). The cells were examined by fluorescent microscopy, scale bars, 25 μm. Data are representative images of each group of cells from three separate experiments.

Figure 6. Calpain-2 silencing mitigates the DTT-up-regulated calpain-2 expression, caspase-12 activation and apoptosis of BRL-3A cells.

BRL-3A cells were transfected with, or without, control or calpain-2 specific siRNA for 48 h and treated with DTT. The relative levels of calpain-2 expression and caspase-12 activation were quantified by Western blot. The percentages of apoptotic cells were quantified by flow cytometry. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments. (A) Calpain-2 silencing. (B) Calpain-2 silencing mitigates the DTT-up-regulated calpain-2 expression. (C). Calpain-2 silencing decreases the DTT-induced caspase-12 activation. (D, E) Calpain-2 silencing mitigates the DTT-triggered apoptosis of BRL-3A cells. $^{*}P<0.05$, $^{**}P<0.01$.