Acta Biochim Biophys Sin 2014, 46: 973–981 | © The Author 2014. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmu091.
Advance Access Publication 30 September 2014

Original Article

Recombinant *Clostridium difficile* toxin B induces endoplasmic reticulum stress in mouse colonal carcinoma cells

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Clostridium difficile **is the main cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans and animals. Its pathogenicity is primarily linked to the secretion of two exotoxins (TcdA and TcdB). Although great progress in the toxic mechanism of TcdA and TcdB has been achieved, there are many conflicting reports about the apoptotic mechanism. More importantly, apoptotic endoplasmic reticulum (ER) stress has been reported in cells treated with Shiga toxins—another kind of cytotoxins that can cause diarrhea and colitis. Herein we checked whether TcdB can induce ER stress. The results showed that recombinant TcdB (rTcdB) activated molecular markers of unfolded protein response, suggesting that rTcdB induced ER stress in CT26 cells. However, rTcdB did not induce the up-regulation of C/EBP homologous protein (CHOP), a classic mediator of apoptotic ER stress, but it activated the precursor of cysteine aspartic acid-specific protease 12 (caspase-12), a controversial mediator of apoptotic ER stress. Besides, glucosyltransferase activity-deficient mutant recombinant TcdB induced ER stress, though it has no cytotoxic or cytopathic effect on CT26 cells. Altogether, these data demonstrated that ER stress induced by rTcdB is glucosyltransferase-independent, indicating that ER stress induced by rTcdB is non-apoptotic. This work also offers us a new insight into the molecular mechanism of CHOP protein expression regulation and the role of CHOP expression in ER stress.**

Keywords *Clostridium difficile*; endoplasmic reticulum stress; apoptosis; unfolded protein response

Received: June 20, 2014 Accepted: August 29, 2014

Introduction

Clostridium difficile is an opportunistic pathogen that flourishes in antibiotic treated and immunocompromised patients and is the leading cause of hospital-acquired diarrhea and pseudomembranous colitis [1,2]. Recently, with the emergence of epidemic strains, *C. difficile* nosocomial infections placed a considerable economic burden on healthcare systems [3,4]. TcdA and TcdB are the major virulence factors of *C. difficile*, and both of them are cytotoxic to most cultured cells. The toxicity of TcdA and TcdB depends on their glucosyltransferase (GT) domain, which targets isoforms of Rho GTPases (Rho A/B/C, Rac1, Cdc42, etc.), resulting in actin condensation and consequent cell rounding, membrane blebbing, and eventual cell death [5]. The pro-apoptotic properties of TcdA and TcdB have been confirmed in different kinds of cell lines, but there are many conflicting reports about apoptotic mechanisms. For example, p53-dependent [6], p53-independent [7], GT-dependent [8], GT-independent [9], and coexisting caspase-dependent and -independent [10] cell death have been extensively studied. So, the precise signaling mechanisms by which TcdA/TcdB induce apoptosis need to be fully characterized. In the past decades, endoplasmic reticulum (ER) stress-induced apoptosis has been reported in a large body of studies, especially in cases of human diseases [11,12]. A recent paper reported that Shiga toxins (Stxs)—a protein family expressed by Gram-negative bacteria *Shigella dysenteriae* serotype 1 and an expanding collection of *Escherichia coli* serotypes—caused diarrhea (bacillary dysentery or hemorrhagic colitis)-induced apoptotic ER stress [13]. However, no related research has been reported in TcdB intoxicated cells until now.

ER is a crucial membranous organelle for normal cell function and development. It plays a fundamental role in synthesis, correct folding, and delivery of proteins to the appropriate cellular destination and calcium homeostasis and storage. Now, it is well documented that various physiological and pathological circumstances can compromise the ER capacity, leading to ER stress. In order to restore the normal ER function, live cells will start a homeostatic signaling network named unfolded protein response (UPR), and

three stress transducer proteins, namely protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) [14], are involved in the initiation of UPR. In resting cells, all these proteins bind to chaperone glucose-regulated protein 78 (BiP/GRP78) to keep them in an inactive state. Once the ER stress happened, BiP dissociates from them, leading to their activation and UPR initiation. If ER stress is not alleviated, apoptotic cell death is promoted [15]. Recently, the functions of ER stress and UPR on cell fate decision have been well documented, and C/EBP homologous protein (CHOP) and caspase-12 have been considered as apoptotic mediators of ER stress [16]. Besides the role of pro-apoptosis, ER stress and UPR have been implicated in other functions, such as innate immunity, metabolism, and cell differentiation [17,18].

Stress reactions have been clearly described in cells treated with TcdA [19,20], and cytosolic calcium rise has also been observed in TcdB intoxicated intact cells [21], so there is a possibility that apoptotic ER stress happened after TcdB exposure. To test this speculation, the expression of some typical ER stress-related genes, the relative molecules of PERK, IRE1α arms of UPR, and two mediators of apoptotic ER stress were investigated in rTcdB or mutant TcdB-treated CT26 cells.

Materials and Methods

Toxins preparation

Plasmids of recombinant TcdB (rTcdB) and mutant recombinant TcdB (mrTcdB with point mutations of W102A and D288N) were provided by Dr Hanping Feng (University of Maryland Dental School, Baltimore, USA). Both plasmids were expressed in *Bacillus megaterium* and purified as described previously [22]. The purity of these recombinant toxins was assessed by gel electrophoresis, and the protein with only a single band on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) was used.

Antibodies, reagents, and cell cultures

The antibodies against BiP, IRE1α, caspase-12, and eIF2α and p-eIF2α were purchased from Cell Signal Technology (Beverly, USA). p-IRE1α and β-actin were purchased from Abcam (Cambridge, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Hangzhou Goodhere Biotechnology Co., Ltd (Hangzhou, China). Dithiothreitol (DTT) was purchased from Invitrogen (Carlsbad, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, trypsin, and other tissue culture reagents were purchased from Gibco (Gaithersburg, USA). 5-Diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, USA). Annexin V-FITC assay kit was purchased from Invitrogen. DNA ladder assay kit was purchased from Applygen Technologies Inc. (Beijing, China).

CT26 cell line was purchased from the American Type Culture Collection (Manassas, USA). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cytotoxic and cytopathic effects of toxins on CT26 cells

The cytopathic effects of rTcdB were examined by cell rounding assays. CT26 cells were seeded in 96-well plate and treated with different doses of rTcdB. Cell rounding was visualized by phase-contrast microscopy. The cytotoxic effect was assessed by MTT assay. Subconfluent CT26 cells (2×10^4 cells/well) seeded in 96-well plate were incubated with rTcdB for 48 and 72 h. MTT (5 mg/ml) was then added (20 μl/well) to each well and the plate was further incubated at 37°C for 4 h. The formazan was solubilized with dimethyl sulfoxide, and absorbance at 490 nm was measured. Cell viability was expressed as the percentage of survival cells compared to untreated cells.

Detection of cell apoptosis

Apoptotic cells were detected by Annexin V apoptosis assay kit and DNA ladder assay kit. Annexin V-FITC apoptosis detection kit was used according to the manufacturer's instructions. Briefly, the cells were treated with 100 ng/ml rTcdB for 6, 12, and 24 h, followed by harvesting and rinsing with phosphate buffered saline. After resuspension in 100 μl of 1× binding buffer containing 1% Annexin V, the cells were incubated for 15 min in the dark. Also, 2 μl of propidium iodide (PI) and 400 μl of 1× binding buffer were then added to the cells and incubated for 5 min before analysis by flow cytometry. Apoptotic cells were shown as the percentage of Annexin V^+/PI^+ and Annexin V^+/PI^- cells.

DNA ladder assay was carried out with a kit (Applygen Technologies Inc., Beijing, China) according to the instructions. The extracted DNA was separated by a 1.8% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

RNA extraction, reverse transcription polymerase chain reaction and real-time polymerase chain reaction

CT26 cells were treated with different doses of rTcdB for 6 h, or treated with 100 ng/ml rTcdB or DTT (2 mM) for the indicated time. After the treatment, the total RNA was isolated using a TRIZOL reagent (Invitrogen). The RNA was then reversely transcribed with PrimeScript® RT reagent kit with gDNA Eraser (DRR047S; Takara, Dalian, China). Real-time polymerase chain reaction (RT-PCR) was carried out by using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara) on ABI 7500 Fast RT-PCR System (Applied Biosystems Inc., Foster City, USA). The specific exon–exon junction primers were designed for RT-PCR. The gene

expression was analyzed by relative quantification using $2^{-\Delta\Delta Ct}$ method by normalizing with actin. Splicing of X-box-binding protein 1 (XBP1) mRNA was examined using the following primers: 5′-TTACGGGAGAAAACTCACG GC-3′ and 5′-GGGTCCAACTTGTCCAGAATGC-3′ [23].

Western blot analysis

Whole-cell extracts were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS), supplemented with protease inhibitor (1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitor (20 mM NaF, 1 mM Na_3VO_4). The proteins were separated on 12% SDS−PAGE and then transferred onto nitrocellulose membranes by electro-blotting. The membranes were blocked with 5% bovine serum albumin. The incubation time for primary or secondary antibodies was 2 h. The protein bands were visualized by an enhanced chemiluminescence assay (Pierce, Rockford, USA). Fold-change values were normalized by β-actin or GAPDH and semi-quantified by gray value measurement using NIH ImageJ software.

Statistical analysis

For the following assays, differences between groups were evaluated for significance by using analysis of variance (ANOVA) followed by Tukey or Dunnett post-hoc test by GraphPad prism 5 software: cell rounding and MTT assay, cell number analysis by flow cytometry, and protein expression or phosphorylation assessment by western blotting. The mRNA expression differences for the UPR genes CHOP, PERK, ATF4, and ATF6 were evaluated by unpaired *t*-test. $P < 0.05$ was considered of significant difference.

Results

Recombinant toxin B expression, purification, and activity test

As shown in **Fig. 1A**, after purification from crude bacterial extracts, single band of rTcdB and mrTcdB protein was observed on SDS−PAGE. The cell rounding assay showed that rTcdB induced cell rounding in both dose- and time-dependent manners, but mrTcdB treated cells showed no obvious morphological change (**Fig. 1B,C**). After exposure to 2 ng/ml rTcdB for 1 h, the ratio of cell rounding was ~50%. When the dose was increased to 100 and 200 ng/ml, almost 90% cells were round within 1 h (**Fig. 1C**).

After 12, 24, 48, and 72 h exposure to indicated dose of rTcdB, all cells showed obvious survival inhibition than untreated cells (**Fig. 1D**). For cells exposed to rTcdB for 12,

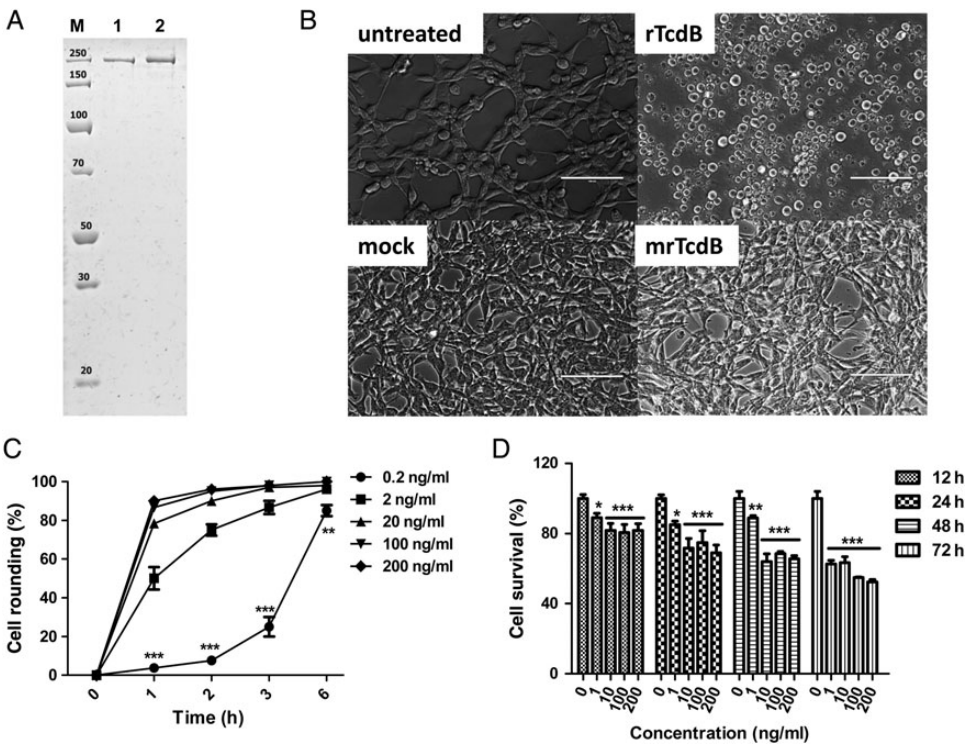

Figure 1. The expression, purification and activity assay of recombinant toxin B (A) Purification of rTcdB and mutant TcdB. M: molecular weight marker; lane 1: purified rTcdB protein; lane 2: purified mrTcdB protein. (B) Cellular morphology before treatment (untreated) or after 36 h treatment of DMEM (mock), rTcdB or mrTcdB. Bar, 100 μm. (C) Cytotoxic effects of rTcdB. CT26 cells in 96-well plate were exposed to various concentrations of rTcdB and then the round cells were counted and the percentages were calculated. The data were the mean ± SD of three independent experiments. (D) Cytopathic effects of rTcdB. CT26 cells in a 96-well plate were exposed to normal medium or indicated doses of rTcdB for 12, 24, 48, or 72 h, and then MTT assays were performed. The data were the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

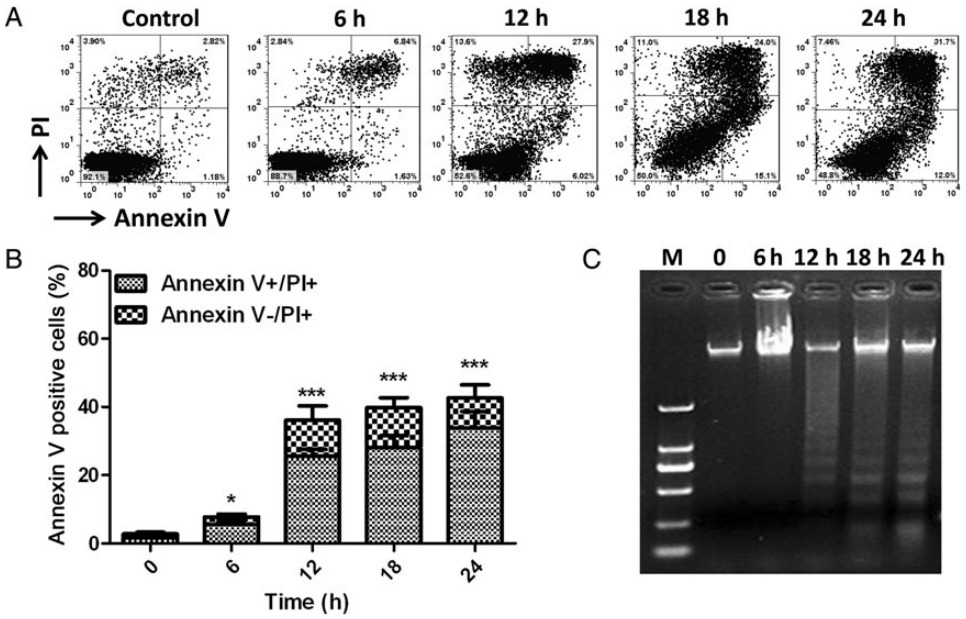

Figure 2. Recombinant toxin B induces apoptosis in CT26 cells (A) Representative result of the apoptosis assay in CT26 cells treated with rTcdB for indicated times. (B) The quantitative analysis for the apoptosis assay. Data were mean ± SD of three independent experiments. * $P < 0.05$, *** $P < 0.001$. (C) Representative result of DNA ladder formation in CT26 cells treated with rTcdB for indicated times. M: 2kb DNA ladder.

24, and 48 h, 1 ng/ml rTcdB treatment showed significant high survival ratio than other doses, and no differences were found among 10, 100, and 200 ng/ml rTcdB treatment (data not shown). For cells exposed to rTcdB for 72 h, the dose of 1 or 10 ng/ml showed significant higher survival ratio than dose of 100 or 200 ng/ml, but no difference was found between the doses of 1 and 10 ng/ml, or 100 and 200 ng/ml (data not shown). In subsequent experiments, 100 ng/ml was selected as working dose because of its excellent capacity in inducing cell rounding within 1 h.

Apoptosis induced in rTcdB-treated CT26 cells

The apoptosis was evaluated by Annexin V positive cells and cells with fragment of DNA. As shown in **Fig. 2A**, ~4% cells were Annexin V positive in untreated group, and after 6, 12, 18, and 24 h treatment of rTcdB, this ratio increased to 8.47%, 33.92%, 39.1%, and 43.7%, respectively. **Figure 2B** shows the means of Annexin V positive cells from three independent experiments. It suggests that 6, 12, 18 and 24 h exposure to rTcdB induced significant increase in the percentage of Annexin V positive cells. Similar results were observed in DNA fragmentation experiment by using the apoptotic DNA ladder kit. As shown in **Fig. 2C**, no DNA fragmentation was detected in control cells, but clear DNA ladder was detected after 12, 18 and 24 h rTcdB treatment.

Expression of molecular markers in UPR after rTcdB treatment

As classic gene markers in ER stress and UPR, mRNA expressions of PERK, CHOP, ATF4, and ATF6 were detected. After rTcdB exposure for 6 h, these four genes showed significant up-regulation in mRNA level (**Fig. 3A**).

BiP protein is a central regulator of ER function. It has been used as a marker of ER stress/UPR in many studies [15]. As shown in **Fig. 3B**,**C**, untreated cells showed low BiP protein expression, and pharmacological agent DTT (inducer of ER stress) induced obvious increase. After rTcdB exposure, BiP protein expression up-regulation started at 3 h, peaked at 12 h, and finally declined at 24 h. It is well known that active PERK induces eIF2α protein phosphorylation, and allows the translation of ATF4 mRNA. As shown in **Fig. 3B**,**C**, elevated p-eIF2α expression was detected after 3 h intoxication, and this elevation continued until the end of the experiment, but no obvious change of eIF2α expression was found during the process. In a word, the up-regulation of PERK and ATF4 mRNA with the up-regulation expression of p-eIF2α together confirmed the activation of PERK branch.

In the three branches of UPR, IRE1 branch is the evolutionarily oldest and the most highly regulated. As an important sensor of UPR, IRE1α has kinase and endonuclease activities [24]. Once ER stress happens, it dimerizes and autotransphosphorylates. Active IRE1α splices a 26-nucleotide intron from the mRNA encoding the transcriptional factor XBP1 (XBP1u) to produce spliced XBP1 (XBP1s) [25]. As shown in **Fig. 3D**, the up-regulation of p-IRE1α was observed after rTcdB exposure for 30 min, 1, 2, and 4 h (top panel), although no significant differences were found compared with untreated cells (lower panel). After 6 h of rTcdB exposure, the expression of p-IRE1α decreased to the control level. Basically, the result of XBP1u splicing was consistent with

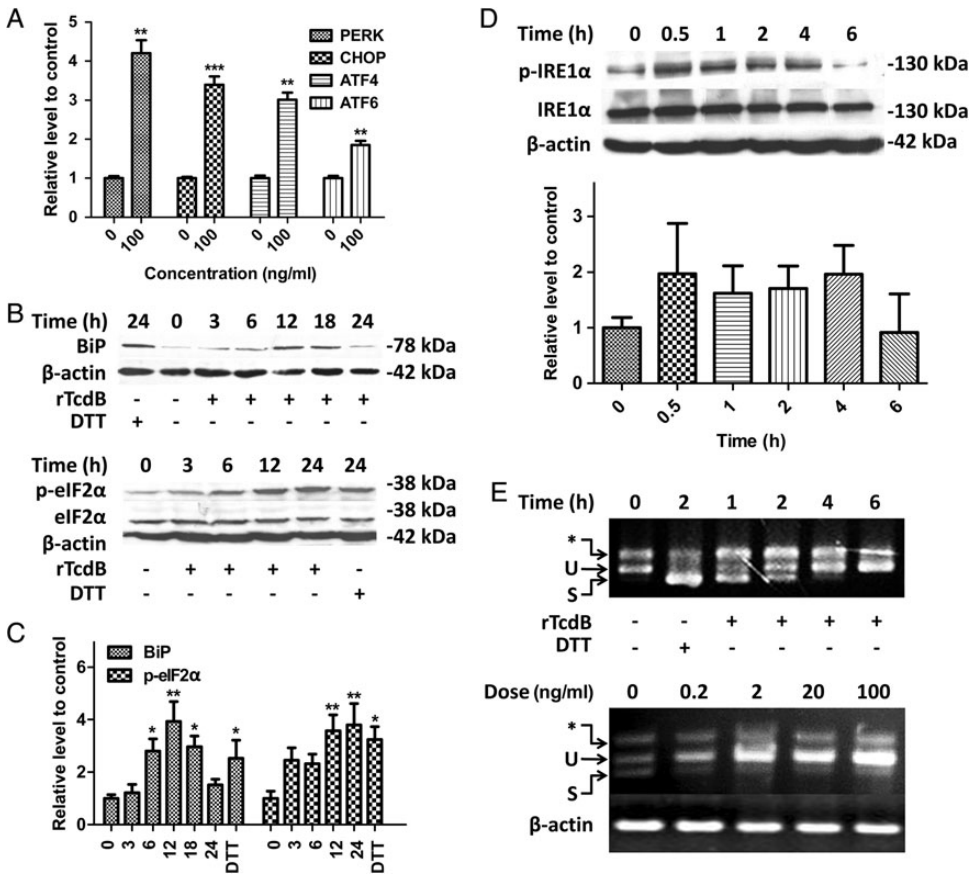

Figure 3. Recombinant toxin B induces expression of molecular markers of UPR (A) The mRNA level changes of CHOP, ATF4, PERK, and ATF6 induced by rTcdB. CT26 cells were untreated (0) or treated with 100 ng/ml rTcdB for 6 h, and the mRNA levels were analyzed by RT-PCR. Fold changes of mRNA level was calculated, and the data were expressed as mean ± SD ($n = 4$). * $P < 0.05$, *** $P < 0.001$ (unpaired *t*-test). (B) BiP/GRP78 (top panel) and p-eIF2α (lower panel) expression by western blotting. CT26 cells were treated with 100 ng/ml rTcdB or 2 mM DTT for the indicated time. Representative results of three independent experiments were shown. (C) The quantitative densitometry of BiP and p-eIF2α. Data were mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ (ANOVA, Dunnett test). (D) The representative result of p-IRE1 expression in CT26 cells treated with rTcdB for the indicated times, and the quantitative densitometry of three independent experiments. (E) The splicing of XBP1 mRNA in CT26 cells treated with 100 ng/ml rTcdB for the indicated times (top panel) (cells treated with DTT for 2 h was used as positive control), or treated with indicated doses of rTcdB for 6 h (lower panel). Splicing of XBP1 mRNA was determined by RT-PCR. Unspliced ('u') and spliced ('s') XBP1 mRNA are indicated. Asterisk identifies a hybrid amplicon resulting from spliced and unspliced XBP1 mRNA.

the result of p-IRE1α expression. As a positive control, the DTT treatment led to the complete splicing of XBP1u mRNA. The obvious splicing of XBP1u mRNA happened after 1 h of rTcdB exposure, and then it attenuated and returned to the baseline after 4 h (**Fig. 3E**, top panel). Interestingly, after 6 h exposure with different doses of rTcdB, the proportion of XBP1u among three bands showed a dose-dependent increase (**Fig. 3E**, lower panel).

ER stress induced by rTcdB is GT activity-independent

Now, the mechanism of apoptotic ER stress is partly understood. The up-regulation of CHOP protein and the processing of caspase-12 have been considered as mediators of apoptotic ER stress [16]. Our results showed that rTcdB treatment induced activation of caspase-12 as evidenced by pro-caspase-12 decrease and appearance of its cleaved fragment (**Fig. 4A**). This activation appeared after rTcdB exposure for 3 h and obvious cleavage of caspase-12 precursor happened after rTcdB exposure for 12 h (**Fig. 4A**). However, the expression of CHOP, a key mediator of apoptotic ER stress, in intoxicated cells was out of expectation (**Fig. 4B**). As expected, CHOP expression was low in untreated cells, but no obvious up-regulation at indicated time was observed in intoxicated cells. By chance, we found that mrTcdB, which was proved to be essentially devoid of GT activity, cytotoxicity and *in vivo* toxicity [26], induced time-dependent up-regulation of CHOP expression in CT26 cells (**Fig. 4C**), and after 24 h of treatment, mrTcdB intoxicated cells showed significant higher CHOP expression than untreated cells or cells treated with rTcdB (**Fig. 4D**). It seemed that GT activity inhibited the expression of CHOP protein. It is well known that GT domain is the biologically activity

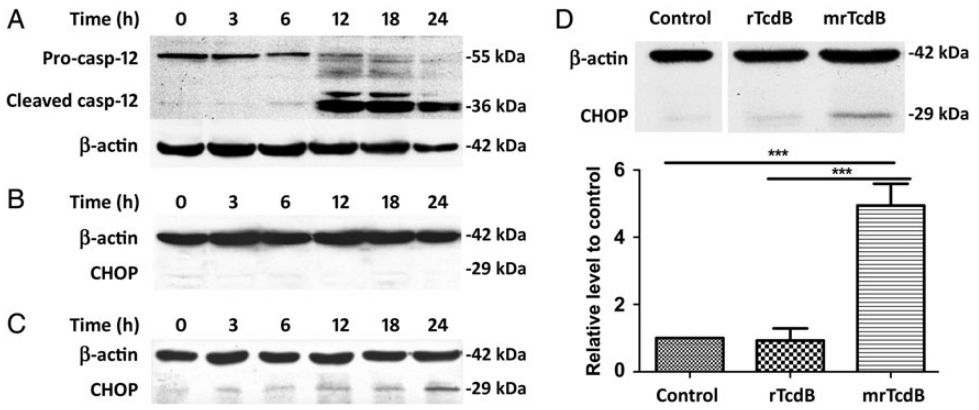

Figure 4. Effect of rTcdB on molecular markers of apoptotic ER stress (A) The representative result of activation of caspase-12 in CT26 cells treated with rTcdB for the indicated times. (B, C) The representative results of CHOP expression in cells treated with rTcdB (B) or mrTcdB (C) for the indicated times. (D) The representative result (top panel) and the quantitative analysis (lower panel) of CHOP expression in cells treated with rTcdB and mrTcdB for 24 h. Data were mean ± SD of three independent experiments. *** $P < 0.001$.

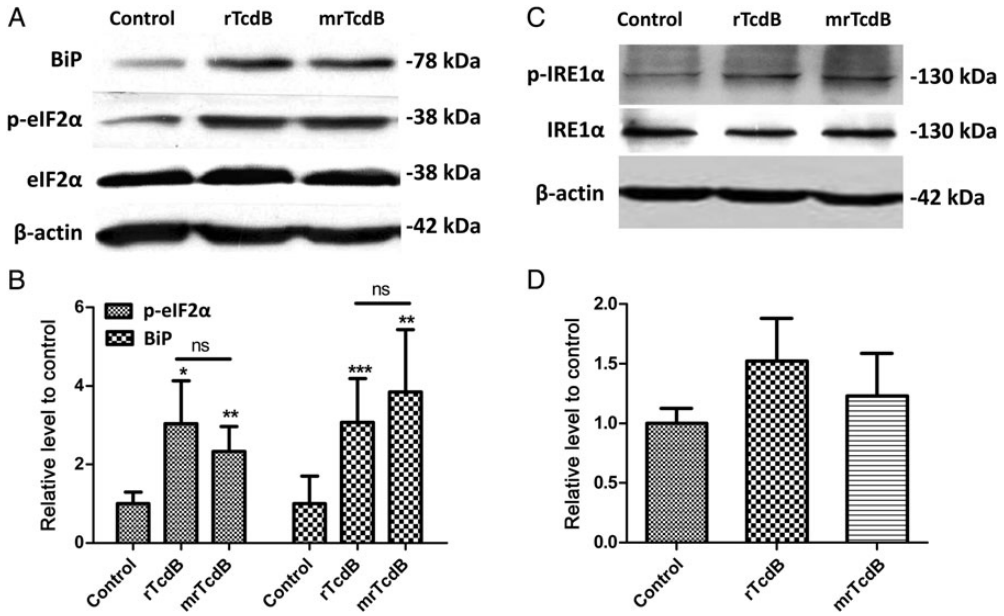

Figure 5. The defect of glucosyltransferase does not influence the up-regulation of BiP, p-eIF2α, and p-IRE1α (A) The representative result of BiP, p-eIF2α, and eIF2α expression in untreated cells or cells treated with rTcdB or mrTcdB for 18 h. (B) The results of quantitative densitometry to untreated cells in **A**. The data were analyzed by ANOVA with Tukey post-hoc analysis. Data were mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, no significant difference. (C) The representative results of p-IRE1α treated with rTcdB or mrTcdB for 2 h. (D) The results of quantitative densitometry to untreated cells in **C**. The data were analyzed by ANOVA with Tukey post-hoc analysis. Data were mean ± SD of three independent experiments.

domain of *C. difficile* toxins [4], but its role in ER stress and UPR regulation induced by rTcdB is unknown. So, the expressions of BiP, p-eIF2α, and p-IRE1α in mrTcdB treated cells were checked. As shown in **Fig. 1B**, the mrTcdB did not cause cell rounding, and both mrTcdB and rTcdB exposure resulted in significant up-regulation of p-eIF2α and BiP protein (**Fig. 5A,B**), and up-regulation of p-IRE1α protein was observed in cells treated with rTcdB or mrTcdB (**Fig. 5C,D**), which demonstrated that GT activity of rTcdB is not necessary for ER stress induction.

Discussion

The connection between bacteria pathogen and UPR has been revealed for a long time [27,28], and recently, several studies proved the induction of apoptotic ER stress in intoxicated mammalian cells with Stxs [13]. In this study, we found that just like Stxs, rTcdB also induced the up-regulation of CHOP, ATF4, and PERK mRNA (**Fig. 3A**). As ATF4 and CHOP are downstream indicators of PERK-eIF2α pathway [29], their up-regulation with

persistent eIF2α phosphorylation (**Fig. 3B**) together indicated the activation of PERK arm of UPR. rTcdB also induced rapid and temporary activation of IRE1α arm as evidenced by the up-regulation of p-IRE1α expression and splicing of XBP1u (**Fig. 3D,E**), and this activation attenuated over time.

Murine caspase-12 is a member of the inflammatory group of the caspase family. Several early study about the caspase-12-deficient mice revealed that it specifically involves in ER stress-induced cell death [30,31], and caspase-12 processing during ER stress-induced apoptosis [32,33] in some cell lines was consistent with this view. However, its dispensable role in apoptosis induced by ER stress has been confirmed by more recent reports [34,35]. In addition, recent studies have suggested that caspase-12 had a more prominent role during the inflammatory and immune responses to bacterial infection [36,37], so caspase-12 processing in rTcdB intoxicated cells (**Fig. 4B**) may play other roles than marker of apoptotic ER stress.

As a key mediator of ER stress-induced apoptosis, CHOP mediates cell death primarily through two mechanisms: alters the transcription of genes involved in apoptosis and oxidative stress [38,39] and relieves the inhibition of protein translation imposed by PERK signaling (via induction of GADD34 expression) through a feedback loop [40]. In this study, it was found that rTcdB induced the up-regulation of CHOP at transcriptional level (**Fig. 2A**), but no obvious up-regulation of protein expression was found during 24 h exposure of rTcdB (**Fig. 4B**), which suggested that the expression of CHOP protein in rTcdB intoxicated cells was controlled at a post-transcriptional level, and CHOP protein is not involved in cell death induced by rTcdB.

In addition, we also found that treatment of mrTcdB which lacks GT activity can induce obvious up-regulation of CHOP protein, which suggested that the GT activity of rTcdB regulated protein expression of CHOP. The more interesting finding is that mrTcdB intoxicated cells showed up-regulation of BiP, p-eIF2α, and p-IRE1α protein (**Fig. 5A,B**), which means that mrTcdB induced ER stress and UPR. As mrTcdB failed to induce cell rounding (**Fig. 1B**) and cell death [26], these results implied that if cells were intoxicated by rTcdB, ER stress is not involved in apoptosis, and CHOP expression is not the mediator of apoptosis induced by ER stress, but its role in mrTcdB intoxicated cells deserved to be further explored.

One recent study reported that caeca and colon tissues of mice which received 10^5 colony-forming units of *C. difficile* VPI10463 vegetative cells showed significantly increased p-eIF2α protein, but neither the splicing of XBP1 nor the up-regulation of ER chaperones was found [41]. Although many studies have shown that the UPR output can be modulated in a context-specific manner [11,42], it was thought that the explanation for the non-full-fledged induction of the UPR is the phosphorylation of eIF2α by a kinase other than by PERK. On the contrary, our study showed that not only significant increase of p-eIF2α protein, but also obvious XBP1 splicing and BiP up-regulation in rTcdB intoxicated cells were found. The possible explanation for the difference is the usage of purified rTcdB and/or the colon carcinoma cell line CT26 in our study. In the report mentioned above, the authors thought that eIF2α phosphorylation was one possible pathway to contain and counteract the damage inflicted on the intestinal epithelium, which may not be true in our model, because both rTcdB and mrTcdB induced the increase of p-eIF2α, but mrTcdB had no detectable cytotoxicity to CT26 cells. So, the exact role of UPR output in our system requires further study.

In summary, our data showed for the first time that rTcdB induced ER stress and UPR *in vitro*, and this induction is GT-independent and non-apoptotic. Besides, it was also found that the GT activity of rTcdB regulated CHOP protein expression, which offers us a new insight into molecular mechanisms of CHOP modulation. Although the expression of CHOP has been reported in all kinds of ER stress, its roles other than apoptosis in the stress response remain unclear, and mrTcdB may be a good tool to explore it. Since induction of CHOP is involved in the development of various diseases, and suppression of CHOP shows a significant therapeutic potential to several diseases models [43,44], our results reveal a potential possibility of rTcdB as a drug to inhibit CHOP protein expression, and further studies on this may provide a basis for new therapeutic approaches to diseases associated with ER stress.

Supplementary Data

Supplementary data are available at ABBS online.

Acknowledgement

We would like to thank Dr Hanping Feng for providing plasmids of rTcdB and mrTcdB for this study, and thank him for his invaluable discussion and comments.

Funding

This work was supported by the grants from the Innovation Foundation for Science and Technology from Department of Education of Guangdong Province (2013KJCX0013), the China Postdoctoral Science Foundation (2012M521593), and the China Postdoctoral Science Foundation (2013T60796).

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