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Possible Involvement of Endoplasmic Reticulum Stress in Hepatotoxicity of Doxorubicin

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Abstract

Hepatic injury caused by doxorubicin (DOX), an anthracycline antitumor agent, is an important dose-limiting adverse event that may restrict treatment dosage and continuation. This study, using HepG2 cells, examines the involvement of endoplasmic reticulum (ER) stress in DOX-induced hepatic damage. The expression level of targets was measured by real-time polymerase chain reaction (PCR) and Western blot analysis. Cytotoxicity was evaluated by the MTT assay. The gene expression of glucose regulated protein 78 (GRP78) was increased in a time-dependent manner after exposure to DOX, and the expression of X-box binding protein 1 (XBP1) and C/EBP homologous protein (CHOP) was increased correspondingly. Concurrently, increased phosphorylation of eIF2α and c-Jun N-terminal kinase (JNK) was observed. Furthermore, the cytotoxic effect of DOX was significantly suppressed by concurrent exposure to 4-phenylbutyric acid(4-PBA), an ER stress inhibitor. This study suggests that DOX induces ER stress and a proapoptotic response, thereby increasing cytotoxicity in Hepatoblastoma G-derived 2 (HepG2) cells, and may contribute to strategies for reducing anthracycline-induced hepatic injury.

Major findings: DOX induced ER stress in HepG2 cells, increasing GRP78, XBP1s, and CHOP expression and phosphorylation of eIF2α and JNK. 4-PBA attenuated DOX-induced cytotoxicity, suggesting that ER stress contributes to DOX-induced hepatic cell injury.

Keywords: Doxorubicin, Endoplasmic Reticulum Stress, HepG2, Hepatotoxicity

1. Introduction

Doxorubicin (DOX) is an anthracycline agent with broad-spectrum antitumor activity. Chemotherapy using DOX plays an essential role in the treatment of cancer; however, it is accompanied by severe adverse events and the emergence of tumour cells resistant to the drug. Although its major adverse effect is cardiotoxicity, many systemic organs are adversely affected by DOX. The hepatotoxicity of DOX has been confirmed in animal models. It has been reported that approximately 30% of patients administered DOX developed liver abnormalities primarily involving hepatocellular injury, as indicated by elevated serum markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin, which may limit dosage and treatment continuation in clinical practice[1]. It is generally considered that oxidative stress is a predominant cause for DOX-induced hepatotoxicity[2]; however, the overall hepatotoxic effects of DOX cannot be fully explained by increased oxidative stress alone. Previous studies demonstrated that endoplasmic reticulum (ER) stress is involved in DOX-induced cellular responses[3,4]; however, its functional significance remains controversial. ER stress has been reported to exert cytoprotective effects through activation of unfolded protein response (UPR) pathways that promote protein folding and cellular adaptation. However, excessive or sustained ER stress can induce apoptosis via pro-apoptotic signalling pathways, including cytoplasmic/element binding

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protein (C/EBP) homologous protein (CHOP) induction and c-Jun N-terminal kinase (JNK) activation. This apparent discrepancy is thought to arise from multiple factors such as differences in the intensity and duration of ER stress, as well as diversity of cellular context. Despite these insights, it is not fully elucidated whether ER stress is consistently induced in hepatocytes under DOX exposure, whether it predominantly contributes to adaptive or apoptotic responses, and which UPR signalling pathways are primarily involved.

In this study, the involvement of ER stress in DOX-induced hepatotoxicity was investigated using HepG2 cells that comprise one of the most widely used models for chemical-associated toxicity tests[5], with a focus on clarifying its functional role and the associated signalling pathways.

2. Materials and Methods

2.1 Chemicals

DOX hydrochloride and HepG2 cells were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and the American Type Culture Collection (Manassas, VA, USA), respectively. All other chemicals were of commercial or analytical grade and were used without further purification.

2.2 Cell Culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Fujifilm Wako Pure Chemical Co., Osaka, Japan) containing 10% fetal bovine serum (Biowest USA, Riverside, MO, USA) and 1% penicillin/streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37°C under a humidified atmosphere of 5% CO_2 in air. Cells between passages 20 and 25 were used in this study. The culture medium was replaced once every two or three days.

2.3 Real-time Quantitative PCR

HepG2 cells were seeded in 10^{-cm} culture dishes at a density of 4×10^6 cells/dish and cultured for 4 days. On day 4 after seeding, the culture medium was replaced with fresh medium containing 2μM DOX, and the cells were cultured for 2 or 4 hr. Control cells were cultured in fresh medium without DOX for the same duration. Total ribonucleic acid (RNA) of HepG2 cells was extracted. RNA purification was performed using a commercial kit purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) according to the instruction manual. After reverse transcription with a commercial kit (GE Healthcare, Seattle, WA, USA), expression levels of targeted genes were analysed by real-time quantitative PCR using SYBR green as the fluorescent dye (Toyobo Co., Ltd., Osaka, Japan). Normalisation of gene expression levels of the target sequence was performed using an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to adjust for variations in the mRNA amount.

2.4 Western Blotting

HepG2 cells were seeded in 10^{-cm} culture dishes at a density of 4×10^6 cells/dish and cultured for 4 days before treatment. On day 4 after seeding, the culture medium was replaced with fresh medium containing 2 μM DOX, and the cells were cultured for 4 hr. Control cells were cultured in fresh medium without DOX for the same duration. Proteins were then extracted from HepG2 cells using Nonidet P (NP-40) lysis buffer containing a protease inhibitor cocktail. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were transferred onto a polyvinylidene fluoride membrane (Bio-Rad Lab., Inc.). The membrane was reacted with specific primary antibodies against spliced X-box binding protein 1 (XBP1; BioLegend, San Diego, CA, USA), CHOP (Proteintech Group, Inc., Rosemont, IL, USA), phosphorylated eukaryotic initiation factor 2 alpha subunit (p-eIF2α; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated JNK (p-JNK; Cell Signaling Technology, Inc., Danvers, MA, USA), and Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH) (Merck, Darmstadt, Germany) for 1 hr at room temperature and then incubated with secondary antibodies for another 1 hr at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) system based on the manufacturer's instructions. The intensity of each target protein was normalised to GAPDH.

2.5 Cell Viability

HepG2 cells were seeded in 12-well plates at a density of 1.5×10^5 cells/well and cultured for 2 days before treatment. On day 2 after seeding, the culture medium was replaced with fresh medium containing DOX in the presence or absence of 5 mM 4-PBA, an ER stress inhibitor, and the cells were cultured for 48 hr. 4-Phenyl butyric Acid (PBA) was

exposed for the same duration as DOX. After treatment, cell viability was evaluated using the MTT assay. MTT solution at a final concentration of 5 mg/mL was added to each well, and the cells were incubated for 15 min. After incubation, the medium was aspirated, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO), followed by measurement of absorbance at 570 nm. Cell viability was normalised to the control value, which was set to 1.

2.6 Statistical Analysis

Data are shown as means ± S.D. Student's unpaired *t*-test was used for comparisons between two groups. In the other statistical analyses, Dunnett's test or the two-tailed multiple *t*-test with Bonferroni correction was applied. A *p*-value of 0.05 or less was considered statistically significant.

3. Results and Discussion

In the present study, the expression level of mRNA for glucose-regulated protein 78 kDa (GRP78) in HepG2 cells was increased in a time-dependent manner after exposure to DOX, and the expression level of protein for XBP1s was correspondingly increased (Figures 1 and 2). Simultaneously, increased phosphorylation of eukaryotic initiation factor 2 alpha subunit (eIF2α) was also observed (Figure 2). eIF2α is phosphorylated through protein kinase RNA-like endoplasmic reticulum kinase (PERK) activation after the occurrence of ER stress[6]. Furthermore, activated inositol-requiring enzyme 1 alpha (IRE1α) causes a frameshift and translation of XBP1s, resulting in protein folding and degradation under ER stress[7]. In this context, our results suggested that DOX induces ER stress via GRP78 expression in HepG2 cells. Importantly, the concomitant upregulation of XBP1s and phosphorylation of eIF2α further suggests activation of UPR, particularly the IRE1α and PERK signalling pathways.

CHOP was shown in the present study to be induced at the transcription process after the exposure of HepG2 cells to DOX, which is consistent with the previous report that upregulated XBP1s caused apoptosis by interacting with binding sites within the promoter of the gene of CHOP[8]. The cascades associated with XBP1s and CHOP were reported to be responsible for the downregulation of Bcl-2 and upregulation of Bcl-2 interacting mediator[9,10], followed by pro-apoptotic ER stress through mitochondrial dysfunction[11]. In addition, increased phosphorylation of JNK was observed in DOX-treated HepG2 cells (Figure 2),

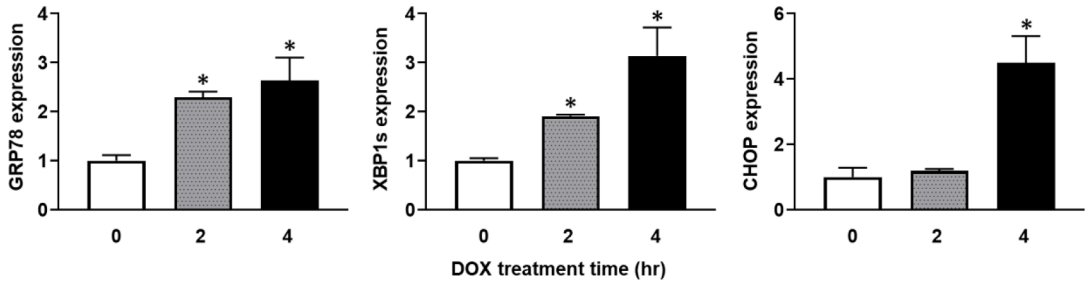


Figure 1. Effects of DOX on ER stress-related mRNA expression in HepG2 cells. Cells were treated with DOX for 2 or 4 hr. Data are presented as means ± S.D. (n=3); **p*<0.05 *vs.* 0 hr.

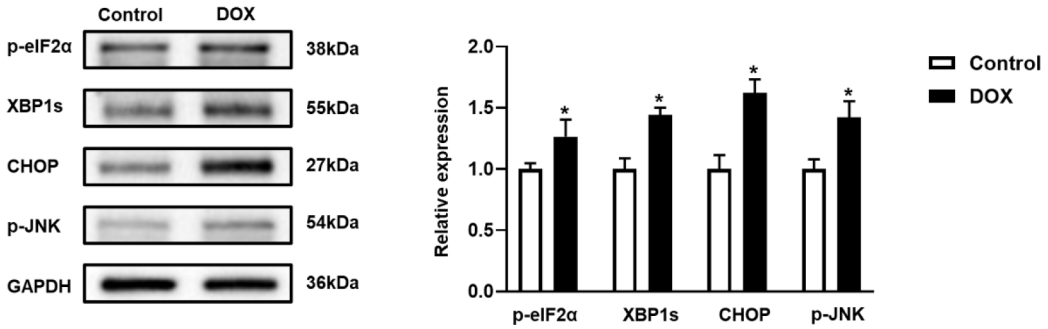


Figure 2. Effects of DOX on ER stress- and apoptosis-related protein expression in HepG2 cells. Cells were treated with DOX for 4 hr. Data are presented as means ± S.D. (n=3); **p*<0.05 *vs.* control.

suggesting enhanced phosphorylation of IRE1α, because JNK is considered to function as a critical mediator linking IRE1-mediated ER stress signalling to mitochondrial apoptotic pathways[12]. Sustained JNK activation has been closely associated with cell death. Thus, the induction of CHOP and the increased phosphorylation of JNK imply that ER stress in this context is not merely adaptive but is associated with the activation of pro-apoptotic signalling. This interpretation is consistent with our previous study demonstrating the induction of intrinsic apoptosis in the livers of DOX-treated mice[13].

The functional significance of DOX-induced ER stress was further substantiated by the observation that co-treatment with 4-phenylbutyric acid (4-PBA), a typical inhibitor of ER stress, markedly attenuated DOX-mediated cytotoxicity in HepG2 cells (Figure 3). Therefore, we suggested that ER stress is likely to play a critical role in mediating hepatotoxicity rather than being a secondary consequence. Consistent with our findings, recent *in vivo* studies in rats have demonstrated that modulation of ER stress profoundly influences the hepatotoxic response to DOX. Gad *et al.*[14] reported that methylene blue confers hepatoprotection against DOX, likely through coordinated suppression of oxidative stress, ER stress, and apoptosis, thereby supporting a causal role for ER stress in mediating liver injury. Similarly, Abdrabou *et al.*[15] showed that suppression of the IRE1α/GRP78/ATF6 signalling may be responsible for a critical role in protection against DOX-induced hepatotoxicity mediated by luteolin, highlighting the broader pathological relevance of ER stress-mediated pathways. On the other hand, the inhibition of ER stress in tumour-bearing mice has been shown to enhance the therapeutic efficacy of DOX by modulating lipid metabolic pathways[16], suggesting that ER stress may exert divergent effects on drug-induced hepatotoxicity and antitumor efficacy. In this regard, we suggest that ER stress serves as a key regulatory hub underlying the dual and opposing effects of DOX on therapeutic outcomes and hepatic injury. Nevertheless, the precise mechanisms by which ER stress modulation influences DOX-induced cytotoxic events remain incompletely elucidated, warranting further investigation.

This study has several limitations that should be considered. Although HepG2 cells are widely used as an *in vitro* model for hepatotoxicity assessment, they do not fully reflect the physiological characteristics of primary hepatocytes, particularly in terms of metabolic capacity and enzyme expression, and therefore the responses observed in this study may not completely represent

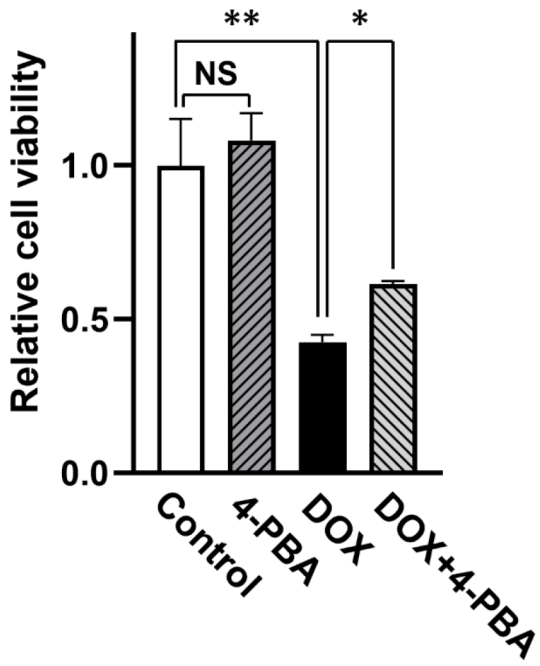


Figure 3. Effects of 4-PBA on DOX-induced cytotoxicity in HepG2 cells. Cells were treated with DOX and/or 4-PBA for 48 hr. Data are presented as means ± S.D. (n=3); *$p<0.05$, **$p<0.01$ *vs.* control.

those in normal liver tissue *in vivo*. In addition, ER stress responses are known to vary depending on cell type and experimental conditions, which may influence the contribution of ER stress to DOX-induced hepatotoxicity. Therefore, caution is warranted when interpreting the present findings, and further studies using primary hepatocytes and *in vivo* models will be necessary to confirm their translational relevance.

4. Conclusion

In conclusion, the present study showed that ER stress was induced by DOX exposure in HepG2 cells, as evidenced by the upregulation of GRP78, activation of XBP1s, and increased phosphorylation of eIF2α and JNK. In addition, the induction of CHOP suggests the involvement of pro-apoptotic signalling pathways associated with sustained ER stress. The attenuation of cytotoxicity by concurrent exposure of 4-PBA further supports the potential contribution of ER stress to DOX-induced cell injury. Taken together, these findings suggest that DOX exposure activates ER stress-associated signalling, which may contribute to cytotoxicity in HepG2 cells, although further studies are required to confirm these mechanisms *in vivo*.

5. References

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Author Contributions

Conceptualisation, S.F.; Methodology, Y.H. and S.F.; Validation, T.M.; Formal analysis, Y.H. and E.I.; Investigation, Y.H. and E.I.; Data curation, Y.H. and E.I.; Writing-original draft preparation, Y.H.; Writing-review and editing, S.F. and H.K.; Visualisation, Y.H.; Supervision, K.N. All authors have read and approved the final manuscript.

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