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High concentraction of taurocholic acid induced apoptosis in HTR-8/SVneo cells via overexpression of ERp29 and activation of p38

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ABSTRACT

Introduction: Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disease associated with a significant risk of fetal complications. Our previous study using an iTRAQ-based proteomics approach showed that ERp29 was overexpressed in the placenta tissue of ICP patients, which was an apoptosis-related protein and has not been investigated in the pathogenesis of ICP. The aim of this study was to explore the role of ERp29 in the mechanism of apoptosis in the placenta of ICP.
Methods: HTR-8/SVneo cells were cultured and treated with different concentrations of taurocholic acid (TCA) (0, 10, 50 and 100 μM). The apoptotic index and cell cycle were detected by flow cytometry; furthermore, the expression levels of ERp29 and p-p38 were detected by western blot. The ERp29-siRNA was also used to confirm the role of ERp29 in TCA induced-apoptosis.
Results: ERp29 expression and the apoptotic index were significantly increased in HTR-8/SVneo cells exposed to 100 μM TCA; so were p-p38 and caspase-3 activity, compared with the 50 μM, 10 μM TCA groups and negative control group ($P < 0.05$, respectively). The induction of apoptosis by TCA and the expression of p-p38 were reduced in HTR-8/SVneo cells after treatment with ERp29-siRNA, compared with controls ($P < 0.05$, respectively).
Conclusions: This study suggested that overexpression of ERp29 may play a key role in TCA-induced apoptosis in HTR-8/SVneo cells via activation of p38, which may participate in the pathogenesis of ICP and may represent a novel target for ICP treatment.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a benign disease for the mother with the characteristic of pruritus and elevated liver enzymes and/or serum bile acids [1,2], but is potentially harmful to the fetus as it is associated with an increased risk of spontaneous preterm delivery, fetal distress and sudden fetal death [3,4]. Up to date, the molecular pathogenesis of ICP and the associated fetal complications still remain elusive.

Studies have demonstrated that the risk of adverse fetal outcomes was associated with the increased levels of maternal bile acids [5], and ursodeoxycholic acid (UDCA) appears to be the most beneficial treatment for patients with ICP, with respect to fetal outcome. Furthermore, studies have also shown that high concentrations of bile acids can induce apoptosis in the placenta, and the incidence of apoptosis decreases after treatment with UDCA, which protects the placenta from the toxic effect of bile acids and commonly used in the management of ICP [3,6]. All these findings suggest that a high concentration of bile acids may play a key role in apoptosis of the placenta and the etiology of fetal complications in ICP patients. However, the exact role and mechanism of bile acids on apoptosis in the placenta of ICP patients has not been clearly identified.

Our previous study using an isobaric tags for relative and absolute quantification (iTRAQ) – based proteomics approach showed significantly increased endoplasmic reticulum protein 29 (ERp29) expression in the placenta of ICP patients compared with controls

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[7]. ERp29 was an apoptosis-related protein and has not been investigated in the pathogenesis of ICP. It's reported that ERp29 is a recently identified endoplasmic reticulum protein and associated with the induction of apoptosis and oxidative stress by regulating p38 expression and phosphorylation [8,9]. Studies have also demonstrated that ERp29 can activate the pathways which mediate caspase activation and apoptosis [10]. Moreover, increased expression of ERp29 was detected in the placenta from women with preeclampsia using proteomics analysis and it may relate to the oxidative stress and apoptosis [11]. The upregulation of ERp29 and increase of apoptosis were also confirmed in ICP patients in our previous study, which suggested that overexpression of ERp29 may play a key role and participate in the induction of apoptosis in the placental tissue of ICP patients. Therefore, the aim of this study was to investigate the role of ERp29 in the pathogenesis of ICP using the trophoblast cell line HTR-8/SVneo in vitro.

2. Materials and methods

2.1. Cell culture and treatment with taurocholic acid

ERp29 was expressed in cytoplasm of placental trophoblast examined by immunohistochemistry in our previous study [7]. Therefore, the trophoblast cell line HTR-8/SVneo was used for further investigation the function of ERp29 and the mechanism of placental apoptosis. The human placenta trophoblast cell line HTR-8/SVneo was a kind gift from Dr Charles H. Graham (Queen's University, Ontario, Canada), and was cultured in RPMI 1640 media supplemented with 10% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co. St. Louis, MO, USA) under a humidified 5% CO_2/95% air atmosphere at 37 °C. After reaching 90% confluence, cells were trypsinized and passaged weekly. The cells were seeded in 6-well plates at a density of 2×10^5 cells per well and treated after overnight culture. Taurocholic acid (TCA) is one of the main bile acids which is elevated in patients with ICP and may be involved in the pathogenesis of ICP [12]. According to Geenes VL's method of TCA treatment in Ref. [6], the cells were divided into four treatment groups, and exposed to different concentrations of TCA (Steraloids, Newport, USA); Concentrations of 10, 50 and 100 μM were added to the culture medium to represent serum bile acid levels observed in normal pregnancy, moderate and severe cholestasis, respectively. Cells treated with an equal volume of vehicle (distilled water) in media were used as negative control (0 μM).

2.2. Cell apoptosis analysis

Following a recent published method which suggested the apoptosis index was significantly increased in placental tissue after treatment with 100 μM TCA for 6 h [6], HTR-8/SVneo cells were treated with TCA at 0 μM, 10 μM, 50 μM and 100 μM for 6 h. HTR-8/SVneo cells were sub-cultured in 6-well plates at a density of 2×10^5 cells per well with complete culture medium overnight, and then exposed to different concentrations of TCA (0 μM, 10 μM, 50 μM and 100 μM) as described above. Apoptotic cells were detected using the Annexin V-FITC apoptosis detection kit I (Shanghai Luanhua Biotechnology, Shanghai, China). Briefly, after treatment with TCA for 6 h, cells were harvested using trypsin, washed with PBS, resuspended in 1 ml binding buffer, and stained with 5 μl Annexin V-FITC and 5 μl PI at room temperature for 10 min. FITC and PI fluorescence was analyzed by flow cytometry (FCM) using a BD FACScan. The assay was performed in triplicate.

2.3. Cell cycle assay

HTR-8/SVneo cells were sub-cultured in 6-well plates at a density of 2×10^5 cells per well with complete culture medium overnight, then washed with PBS and starved in serum-free RPMI 1640 media for 24 h to synchronize the cell cycle. The cell cycle was initiated by replacement of the starvation medium with full medium (RPMI 1640 media containing 10% FBS) supplemented with different concentrations of TCA (0 μM, 10 μM, 50 μM and 100 μM) for various times (24 h, 48 h and 72 h). Cells were harvested, washed twice with PBS, centrifuged, fixed in 70% ethanol and stained with 500 μl PI solution (100 μg/ml RNase and 50 μg/ml PI in 1 × PBS). The percentage of cells distributed in each phase of the cell cycle (G1, S and G2/M) was quantified by FCM using a BD FACScan; data was analyzed using Cell Quest software. The assay was performed in triplicate.

2.4. Analysis of ERp29 and phosphorylated (p)-p38 expression in HTR-8/SVneo cell lines

Studies suggested that ERp29 was associated with apoptosis by regulating p38 phosphorylation [8,9]. In order to further investigate the mechanisms of ERp29 involved in apoptosis in ICP, HTR-8/SVneo cells were treated with TCA at 0 μM, 10 μM, 50 μM and 100 μM for 6 h and the expression levels of ERp29 and p-p38 expression were measured by western blot. Briefly, after treatment with TCA for 6 h, cells were then harvested, washed with PBS, homogenized with an ULTRA TURRAX homogenizer (Ika, Petaling Jaya, Malaysia) in a lysis buffer that contained 7 M urea, 2 M thiourea, 2% (w/v) DTT, 1% (v/w) Protease Inhibitor Cocktail Kit (Pierce Biotechnology, Rockford, IL, USA), at 11,000 IU/min on ice (10 bursts of 10 s, each interspersed with short pauses). Suspensions were shaken at 4 °C for 1 h, and insoluble molecules were removed by centrifugation at 40000 × g at 4 °C for 1 h. The protein concentration in each sample was determined by the Bradford method using BSA as the standard. Samples containing 50 μg of protein were electrophoresed on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare, San Francisco, CA, USA). The membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat milk powder for 1 h, and incubated overnight with anti-ERp29 antibody (ab42002, 1:150; Abcam, Cambridge, MA, USA) and p-p38 (1:500; Abcam) diluted in TBS/5% non-fat milk powder. Tubulin was used as a loading control. The membranes were washed three times (10 min each) with TBS and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000; Beijing ZhongShan Biotechnology, Beijing, China). Specific proteins were detected using an ECL kit and AlphaImager (FluorChem5500; Alpha Innotech). The protein expression levels were analyzed using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). This assay was validated in triplicate.

2.5. Caspase-3 activity assay

Caspase-3 activity, one of the apoptosis markers was also measured in HTR-8/SVneo cell line after treatment with TCA at 0 μM, 10 μM, 50 μM and 100 μM, for 6 h. HTR-8 cells were seeded in 6-well culture plates and incubated in RPMI 1640 supplemented with 10% FBS. After being treated with different concentrations of TCA (0 μM, 10 μM, 50 μM and 100 μM) for 6 h, protein extracts were prepared and Caspase-3 activity was measured using Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, 10 μl protein extracts and 80 μl reaction buffer were mixed with 10 μl Ac-DEVD-pNA substrate and then incubated at 37 °C for 2 h. Samples were measured with an ELISA Reader (Bio-Rad instrument Group, Hercules, CA, USA) at an absorbance of 405 nm. The detail analysis procedure was described in the manufacturer's protocol (Beyotime Institute of Biotechnology).

2.6. Small interference RNA (siRNA) transfection

HTR-8/SVneo cells were sub-cultured in 6-well plates at a density of 2×10^5 cells per well with complete culture medium overnight and transfected with siRNA for ERp29 (Genepharma, Shanghai, China) using Lipofectamine 2000 transfection reagent according to the protocol provided by the manufacturer. After 24 h, media was exchanged and the cells were treated with TCA (100 μM) for an additional 6 h. Transfection efficiency of ERp29 siRNA was assessed by western blot. Finally, cells were harvested for apoptosis assays and Western blot analysis as described above.

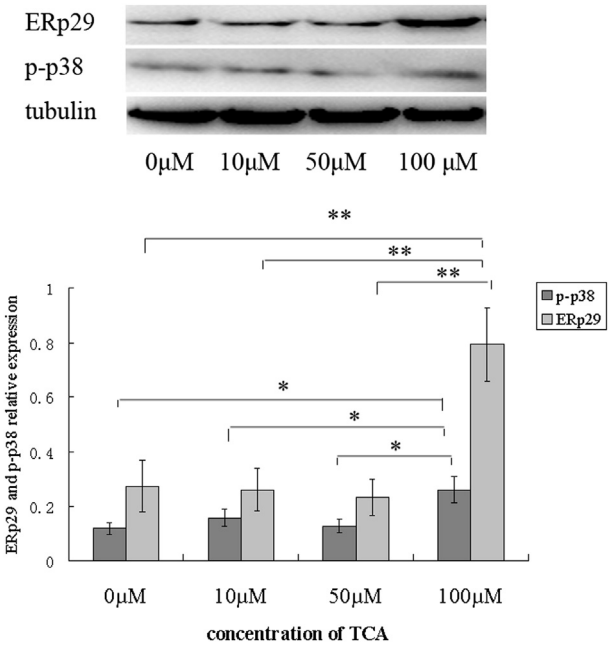

Fig. 1. The expression levels of ERp29 and p-p38 in HTR-8/SVneo cells by the treatment with TCA ERp29 and p-p38 expression levels were significantly increased in HTR-8/SVneo cells after treatment with TCA at 100 μM compared to treatment with TCA at 0 μM, 10 μM or 50 μM quantified by western blotting, respectively. Each assay was validated in triplicate. ($^*P < 0.05$; $^{**}P < 0.01$).