Involvement of gap junctions in propylthiouracil-induced cytotoxicity in BRL-3A cells

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Abstract. Gap junctions (GJs), which are important plasma membrane channels for the transfer of signaling molecules between adjacent cells, have been implicated in drug-induced liver injury. However, the influence and the underlying mechanisms of GJs in propylthiouracil (PTU)-induced hepatotoxicity are unclear. In the present study, distinct manipulations were performed to regulate GJ function in the BRL-3A rat liver cell line. The results indicated that the toxic effect of PTU in BRL-3A cells was mediated by GJ intercellular communication, as cell death was significantly attenuated in the absence of functional GJ channels. Furthermore, the specific knockdown of connexin-32 (Cx32; a major GJ component protein in hepatocytes) using small interfering RNA was observed to decrease necrosis, intracellular PTU content and the level of reactive oxygen species (ROS) following PTU exposure. These observations demonstrated that suppressing GJ Cx32 could confer protection against PTU-induced cytotoxicity through decreasing the accumulation of PTU and ROS. To the best of our knowledge, the present study is the first to demonstrate the role and possible underlying mechanisms of GJs in the regulation of PTU-induced toxicity in BRL-3A rat liver cells.

Introduction

The Food and Drug Administration (FDA) approved propylthiouracil (PTU) for the treatment of Graves' disease in 1947 (1). In nearly 70 years of clinical application, reports of PTU-associated liver injury and failure, and even fatality, have accumulated for adult and pediatric patients (2-6). A warning regarding the potential risk of severe hepatic injury associated with PTU was issued by the FDA in 2009 (7). Therefore, it is recommended that patients receiving PTU therapy have their liver function closely monitored. PTU-induced liver injury primarily manifests as differing degrees of hepatocyte necrosis (8); however, the underlying mechanisms are largely unknown.

Gap junctions (GJs) directly connect the cytoplasm of adjacent cells, mediating the intercellular transmission of signaling molecules. Six transmembrane connexin (Cx) monomers are arranged in a circle to form a hemichannel, and then two hemichannels from neighboring plasma membranes are docked to form the GJ (9,10). Cx expression is distinct in a variety of tissues, and Cx32 is the major GJ protein in hepatocytes (11,12).

GJ-mediated intercellular communication (GJIC) is involved in a number of physiological and pathological processes (13-15). Previous reports have suggested a role for GJ channels in drug-induced liver injury (DILI) (16-18). Downregulation of GJs composed of Cx32 (Cx32-GJs) could reduce the hepatotoxicity of acetaminophen, D-galactosamine and carbon tetrachloride (19,20). Likewise, propofol protects rat liver cells from sevoflurane-induced cytotoxicity through inhibiting GJ channels (21). Based on this evidence, the inhibition of hepatic Cx32-GJs could prove to be an effective strategy for controlling DILI. However, whether this GJ-mediated hepatoprotection is effective against PTU toxicity, and the potential underlying mechanism of this, remain unknown. In the present study, the role and underlying mechanisms of GJs in PTU-induced toxicity were explored in BRL-3A cells.

Materials and methods

Materials. PTU, carbenoxolone (CBX), anti-GAPDH and secondary antibodies for western blotting were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-Cx32 antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Cell culture reagents, Lipofectamine 2000 and calcine acetoxyethyl ester (Calcein-AM) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Cell Counting kit-8 (CCK-8) was obtained from Dojindo (Mashikimachi, Kumamoto, Japan). The 2',7'-dichlorofluorescin diacetate (DCFH-DA) was from Beyotime Institute of Biotechnology (Haimen, China).
All other reagents and chemicals were obtained from Sigma-Aldrich; Merck KGaA, unless otherwise stated.

**Cell culture.** The BRL-3A rat liver cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin at 37°C in an atmosphere containing 5% CO₂.

**Detection kit (GE Healthcare Life Sciences, Little Chalfont, UK)** visualized using an Amersham Enhanced Chemiluminescence

**Temperature** for 1 h. The immunopositive protein bands were conjugated; A4416; dilution, 1:4,000) were then added at room temperature for 3 h. Secondary antibodies (goat anti-mouse IgG-peroxidase 1:1,000) and GAPDH (G8795; dilution, 1:2,000) overnight with specific antibodies against Cx32 (sc-59948; dilution, 1:1000) and the intensities were detected by the Quantity One software (Bio-Rad, version 4.6.2).

‘Parachute’ dye-coupling assay. A ‘parachute’ dye-coupling assay was performed to evaluate GJ function as previously described (26). Cells were seeded into 12-well plates and cultured to 80-90% confluency. Donor cells from one well were labeled with 5 µM Calcein-AM and trypsinized, diluted and seeded onto receiver cells at a ratio of 1:150 (donor/receiver). GJs formed between donor cells and receiver cells during a 4-h incubation at 37°C and were monitored using an Olympus IX71 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Under each experimental condition (treatment of CBX or (si)RNA transfection), the average number of receiver cells containing calcein dye per donor cell was counted and normalized to that of the vehicle control.

**Observation of cell morphology.** Cell morphology was observed using a FEI Quanta-400 scanning electron microscope (SEM; Thermo Fisher Scientific, Inc.). Sterile slides were placed in 12-well plates and served as substrates. Cells were treated with vehicle control or PTU at 0.6 and 0.8 mg/ml for 24 h, rinsed with PBS and fixed in 4% paraformaldehyde for 1 h at room temperature. Following gradient dehydration in a series of ethanol from 30-100%, the samples were dried in a vacuum freeze-drying apparatus for 1 h and were treated with gold sputtering for SEM observation.

**Detection of PTU concentration in BRL-3A cells.** Reversed-phase high-performance liquid chromatography was adopted to measure the PTU content in BRL-3A cells (27). Briefly, following incubation with PTU at 0.6 and 0.8 mg/ml for 24 h, cells were washed with PBS three times, harvested by trypsinization, resuspended in PBS and counted. Five freeze-thaw cycles were used to lyse the cells. Methanol (HPLC-grade) was added for protein precipitation. Following centrifugation at 14,167 x g for 10 min at 4°C, the supernatant was collected and injected into a Shimadzu LC-20AD system using Lipofectamine 2000, according to the manufacturer’s instructions. After 48 h incubation, western blotting and a ‘parachute’ assay, as described below, were performed to confirm Cx32 expression knockdown and GJIC inhibition.

**Small interfering (si)RNA transfection.** Cx32 expression was inhibited by siRNA transfection in BRL-3A cells. The siRNA sequences targeted against the rat Cx32 gene were as described previously (23,24): siRNA-1, 5'-CACCAACAACACATAGAAA-3'; siRNA-2, 5'-GCACTCGCATTTCCCTCA-3'; and siRNA-3, 5'-GCTTCTACCTGATACATAA-3'. Cx32 siRNAs (50 nM) or the negative control siRNA (NC siRNA) were transiently transfected into BRL-3A cells using Lipofectamine 2000, according to the manufacturer's instructions. After 48 h incubation, western blotting and a ‘parachute’ assay, as described below, were performed to confirm Cx32 expression knockdown and GJIC inhibition.

**Western blotting.** The procedure of western blotting was performed as described previously (25). In brief, cell lysates were obtained in lysis buffer (P0013; Beyotime Institute of Biotechnology), sonicated and centrifuged at 14,167 x g at 4°C for 30 min. Protein concentration was determined by the BCA assay. Cell lysates (20 µg per lane) were separated using SDS-PAGE in 10% Tris-glycine gels and transferred to nitrocellulose membranes. Following blocking with 5% skimmed dry milk in Tris buffered saline with Tween-20 (0.05% Tween-20) at room temperature for 1 h, the membranes were incubated with specific antibodies against Cx32 (sc-59948; dilution, 1:1000) and GAPDH (G8795; dilution, 1:2000) overnight at 4°C. Secondary antibodies (goat anti-mouse IgG-peroxidase conjugated; A4416; dilution, 1:4000) were then added at room temperature for 1 h. The immunopositive protein bands were visualized using an Amersham Enhanced Chemiluminescence Detection kit (GE Healthcare Life Sciences, Little Chalfont, UK) and the intensities were detected by the Quantity One software (Bio-Rad, version 4.6.2).

**Detection of PTU concentration in BRL-3A cells.** Reversed-phase high-performance liquid chromatography was adopted to measure the PTU content in BRL-3A cells (27). Briefly, following incubation with PTU at 0.6 and 0.8 mg/ml for 24 h, cells were washed with PBS three times, harvested by trypsinization, resuspended in PBS and counted. Five freeze-thaw cycles were used to lyse the cells. Methanol (HPLC-grade) was added for protein precipitation. Following centrifugation at 14,167 x g for 10 min at 4°C, the supernatant was collected and injected into a Shimadzu LC-20AD system (Shimadzu Corporation, Kyoto, Japan). The chromatographic conditions used were as follows: Application of a Luna C8 column (250x4.6 mm; 5mm: Phenomenex, Torrance, CA, USA), methanol and water (40:60) was used as the mobile phase at a flow rate of 1.0 ml/min, which was detected at a wavelength of 272 nm with a column temperature of 30°C. The concentrations of PTU in the cell samples were calculated using a calibration curve method.

**Analysis of intracellular reactive oxygen species (ROS) levels.** Following exposure to PTU at 0.6 and 0.8 mg/ml for 6 h, BRL-3A cells were labeled with DCFH-DA, which is hydrolyzed by esterase into DCFH without fluorescence. Intracellular ROS can oxidize non-fluorescent DCFH to fluorescent DCF (28). Formation of DCF was determined using a Perkin LS55 fluorescence spectrophotometer (PerkinElmer Inc., Waltham, MA, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Fluorescence intensity was normalized to that of the vehicle control and was regarded as a measure of the intracellular ROS level. The DCF images were captured via fluorescence microscopy (Olympus IX71; Olympus Corporation).
Statistical analysis. The data were presented as the mean ± standard error and were analyzed using IBM SPSS Statistics 19.0 (Armonk, NY, USA). Statistical analysis was performed by Student's t-test or one-way analysis of variance followed by Dunnett's test. In all cases, P<0.05 was considered to indicate a statistically significant difference.

Results

Cell density influences the cytotoxic effect of PTU in BRL-3A cells. BRL-3A cells were cultured separately under two cell-density conditions for initial investigation of the influence of GJIC on PTU toxicity. At a high cell density (2x10^5 cells per cm^2), GJs formed efficiently as the cells had frequent contact with one another. At a low cell density (5x10^4 cells per cm^2), the wide dispersion of cells infrequently permitted the formation of GJs. A preliminary cell viability assay for concentrations screening was performed with PTU from 0.3 to 0.8 mg/ml (data not shown). The concentrations of 0.6 and 0.8 mg/ml PTU were chosen in the present study. PTU-induced cytotoxicity was assessed using a CCK-8 assay and a standard colony-formation assay. Fig. 1 illustrates that PTU treatment, at concentrations of 0.6 and 0.8 mg/ml, decreased the cell survival of BRL-3A cells under both culture conditions. However, the survival was significantly greater in the low-density condition compared with the high-density condition. Thus, the toxicity of PTU in BRL-3A cells was reduced under low cell-density conditions wherein there was a deficiency in GJIC.

Pharmacological inhibition of GJ function decreases PTU cytotoxicity in BRL-3A cells. To investigate whether the cell density-dependence of PTU toxicity in BRL-3A cells is associated with GJIC, an extensive GJ inhibitor, CBX (29), was adopted to manipulate GJ function. BRL-3A cells were pretreated with the CBX prior to exposure to PTU. In a subsequent ‘parachute’ assay, dye-coupling was significantly blocked by CBX pretreatment (Fig. 2A). Under high cell-density culture conditions, the cell survival was significantly increased following pretreatment with 100 µM CBX for 1 h at PTU concentrations of 0.6 and 0.8 mg/ml, as compared with PTU alone (Fig. 2B and C). These results indicate that the inhibition of GJs contributes to the decreased toxicity of PTU at high BRL-3A cell densities.

Suppression of Cx32-GJ function by Cx32 knockdown attenuates PTU cytotoxicity. Cx32 is a key GJ constituent protein in liver cells (12). Specific knockdown of the Cx32 gene was conducted to confirm the effect of Cx32-GJ on PTU toxicity. As indicated in Fig. 3A and B, the downregulation of Cx32 expression significantly suppressed the spread of calcein dye through GJs in siRNA-transfected cells. Furthermore, Cx32-knockdown (siRNA-1 transfection) increased the cell viability by factors of 1.31 and 1.29 in the presence of 0.6 and 0.8 mg/ml PTU, respectively, at high cell densities (Fig. 3C). Likewise, clonogenic capacity was improved at these two concentrations when Cx32-GJs were inhibited (Fig. 3D). The results indicate that Cx32-GJ serves an important role in the PTU-induced cytotoxicity of BRL-3A cells.

Cx32-GJs influence cell morphology and necrosis. PTU has been demonstrated to induce necrosis in liver injury (8). To assess the necrosis of BRL-3A cells subjected to PTU in the absence of Cx32-GJ, SEM images were captured. Fig. 4 illustrates the typical morphological characteristics of cell necrosis, including cell swelling and spillovers of cellular content (as indicated by the arrows), which were clearly noted following PTU treatment, but were less obvious following siRNA-mediated Cx32-GJ inhibition. These results suggest that the suppression Cx32-GJ attenuates PTU hepatotoxicity, which is partly associated with a decrease in PTU-induced necrosis.

Inhibition of Cx32-GJ decreases intracellular PTU accumulation. GJs facilitate direct intercellular communication between neighboring cells. PTU, as a small molecule, could be transmitted through GJs. To assess whether the PTU concentration in BRL-3A cells could be reduced by blocking Cx32-GJ function, the intracellular PTU content was determined. As indicated in Fig. 5, the level of cellular PTU [PTU (µg) per 10^5 cells] significantly decreased when cells were transfected with Cx32 siRNA-1. The reduction of PTU uptake in BRL-3A cells between those with and without Cx32-GJ were significant at 0.6 and 0.8 mg/ml PTU. These results demonstrate that PTU accumulation is reduced when Cx32-GJ is downregulated.
Figure 2. Effect of CBX on PTU cytotoxicity in BRL-3A cells. (A) CBX suppressed the spread of dye between BRL-3A cells, as indicated by a ‘parachute’ assay (magnification, x100). *P<0.05 vs. vehicle control. (B) Cell viability by Cell Counting kit-8 and the (C) survival fraction as well as the representative colony images by a standard colony-formation assay of BRL-3A cells incubated with 0.6 and 0.8 mg/ml PTU at a high cell density following pretreatment with CBX. *P<0.05 vs. 0.6 mg/ml PTU with GJ; #P<0.05 vs. 0.8 mg/ml PTU with GJ. CBX, carbenoxolone; PTU, propylthiouracil; GJ, gap junction.

Figure 3. Effect of Cx32 siRNA-mediated inhibition of GJ function on PTU cytotoxicity in BRL-3A cells. (A and B) Western blot and ‘parachute’ dye-coupling assays (magnification, x100) were adopted to measure the influence of siRNA on Cx32 expression and the degree of GJ intercellular communication. *P<0.05 vs. control. (C) Cell viability of BRL-3A cells exposed to PTU for 24 h and (D) survival fraction and the representative colony images of BRL-3A cells exposed to PTU for 12 h, each at a high cell density, following transfection with Cx32 siRNA-1. *P<0.05 vs. 0.6 mg/ml PTU with GJ; #P<0.05 vs. 0.8 mg/ml PTU with GJ. CBX, carbenoxolone; PTU, propylthiouracil; GJ, gap junction; siRNA, small interfering RNA; Cx32, connexin 32.
Inhibition of Cx32-GJ prevents ROS transmission. To investigate the possibility of ROS transmission through GJs, intracellular ROS levels in the presence and absence of Cx32-GJ were detected. The results revealed that PTU-induced toxicity was accompanied by an increase in ROS production at concentrations ≤0.8 mg/ml. Fig. 6 illustrates that Cx32 siRNA-1 transfection markedly suppressed the PTU-induced increase in DCF fluorescence, which was quantified by fluorescence spectroscopy. The levels of intracellular ROS were significantly reduced in the Cx32-knockdown group at PTU concentrations of 0.6 and 0.8 mg/ml. These findings suggest that the attenuation of PTU-induced toxicity by Cx32-GJ downregulation may be associated with the inhibition of ROS transmission.

Discussion

In the present study, it was demonstrated that PTU-induced toxicity depends on GJs in BRL-3A cells. The investigation indicated that the inhibition of GJs could protect against PTU-induced liver cell damage in vitro. Furthermore, inhibition of Cx32-GJ prevents ROS transmission. To investigate the possibility of ROS transmission through GJs, intracellular ROS levels in the presence and absence of Cx32-GJ were detected. The results revealed that PTU-induced toxicity was accompanied by an increase in ROS production at concentrations ≤0.8 mg/ml. Fig. 6 illustrates that Cx32 siRNA-1 transfection markedly suppressed the PTU-induced increase in DCF fluorescence, which was quantified by fluorescence spectroscopy. The levels of intracellular ROS were significantly reduced in the Cx32-knockdown group at PTU concentrations of 0.6 and 0.8 mg/ml. These findings suggest that the attenuation of PTU-induced toxicity by Cx32-GJ downregulation may be associated with the inhibition of ROS transmission.

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PTU could markedly suppress glutathione S-transferase (GST) activity to interfere with the antioxidation of glutathione (41). Therefore, the present study was performed to investigate the possibility that PTU is a GJ-permeable ‘injury signal’. The results indicated that PTU uptake was decreased in cells deficient in functional Cx32 channels. This decline may not only be conducive to the restoration of GST activity; it may also weaken allergic responses caused by PTU acting as a hapten.

Oxidative stress was observed in hypothyroid rats induced by administration of drinking water containing 0.05% PTU for 8 weeks (42,43). Oxidative stress is an important pathophysiological basis of liver injury, in which ROS is the essential mediator; it induces lipid peroxidation of the cellular and mitochondrial membranes, causing changes in permeability and affecting signal transduction pathways (e.g., ROS/Protein kinase C, ROS/ c-Jun N-terminal kinase (44-46). Previous studies identified that ROS can spread through Cx32 channels, acting as toxic molecules (19,47). The experimental results in the present study suggested that ROS induced by PTU-exposure were regulated by Cx32-GJs. Rat hepatocytes deficient in Cx32 could prevent the passage of ROS between cells. Thus, ROS could be PTU-induced ‘injury signals’ with selective Cx32 channel permeability.

Immortalized BRL-3A cells endogenously expressing Cx32 and Cx43 is a simple model for investigating the role of GJ in PTU-induced hepatotoxicity in vitro. However, the limitations of using only one rat liver cell line are the differences in species and primary hepatocytes (such as confounding factors arising from the complicated hemodynamic system), the effects of GJs on the PTU-induced cytotoxicity in human hepatocytes, and in vivo, remain to be explored.

In conclusion, the role of GJs in PTU-induced cytotoxicity was investigated in BRL-3A cells. The results indicated that the suppression of Cx32-GJ channels protected against PTU-cytotoxicity through reducing necrosis. Furthermore, PTU and ROS appeared to affect the Cx32-GJ-mediated hepatoprotective effect, acting as ‘injury signals’.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

NT designed the current study. NT, ZC, HC, LC and BC performed the experiments. NT, ZC and BL analyzed the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.
The authors declare that they have no competing interests.

Competing interests

Patient consent for publication

Not applicable.

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