Dysregulation of Na\textsuperscript{+}-taurocholate co-transporting polypeptide (NTCP) plays a vital role in isoniazid-induced HepG2 cells apoptosis

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Abstract: Isoniazid (INH) remains a mainstay for the prevention and treatment of tuberculosis though it can cause liver injury and even liver failure. Therefore this study aimed to investigate the mechanism through which INH causes hepatotoxicity based on liver transporter Na\textsuperscript{+}-taurocholate co-transporting polypeptide (NTCP) using human hepatocellular carcinoma (HepG2) cell line. We evaluated cytotoxicity induced by INH and determined the role of NTCP in INH-induced HepG2 cells apoptosis. We discovered that after 24 h treatment of 6.5, 13, 26, 52 and 104 mM INH, the cell viability decreased in a concentration-dependent manner. NTCP was increased at 6.5, 13, 26 and 52 mM INH, and declined to normal level at 104 mM INH. Next NTCP inhibitor Cyclosporin A (CsA) blocked the cell viability suppression induced by INH. Likewise, the tendency to increase Annexin-positive cells and cleaved-caspase3 expression in response to INH was dependent on NTCP. Furthermore, CsA also partly abolished significant alterations including mitochondrial membrane potential and ratio of Bcl-2/Bax expression caused by INH. At last, we identified hepatocyte nuclear factor 4α (HNF4α)/peroxisome proliferator activated receptor γ coactivator 1α (PGC1α) as potential regulators of NTCP expression. Briefly, the results above provided the first evidence that NTCP was indispensable in INH-induced HepG2 cells apoptosis and perhaps regulated by HNF4α/PGC1α.

Keywords: Isoniazid, Na\textsuperscript{+}-taurocholate co-transporting polypeptide, HepG2 cells, apoptosis, hepatocyte nuclear factor 4α, peroxisome proliferator activated receptor γ coactivator 1α

Introduction

Drug-induced liver injury (DILI) is a serious adverse drug reaction and contributes greatly to the acute liver failure and transplant, which leads to the withdrawal of pharmaceuticals from clinical use [1]. Interest in drug-induced liver injury (DILI) has dramatically increased over the past decade, among the various mechanisms of liver injury including hepatocellular, cholestatic and mixed types of liver damage, the accumulation of bile acids (BAs) within hepatocytes is thought to be a primary mechanism for the development of DILI [2]. In the basolateral hepatocyte membrane, uptake of bile salts occurs predominantly in a sodium-dependent manner via the sodium taurocholate cotransporting polypeptide (Ntcp in rodents, NTCP in humans) [3]. It has also been reported NTCP is a transporter of steroidal hormones and a variety of drugs [4, 5]. Upregulation of NTCP leads to cholestasis and accumulation of toxic products, which is closely concen-
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Notably, the current two reports about mechanism of INH-induced DILI in vivo is controversial. Guo et al. showed that expressions of Ntcp were inhibited in rat liver treated with INH (100 mg/kg) and the downregulated expression of hepatic Ntcp might play an important role in the development of anti-TB drugs induced liver injury [14]. In contrast, Zhou et al. reported that Ntcp expression was increased in rat liver treated with RIF (60 mg/kg) and INH (60 mg/kg) [15]. To our knowledge, except the two contrast studies in rat, few reports explaining cellular and molecular mechanism underlying INH-induced hepatotoxicity based on NTCP are available. Therefore a better understanding in human origin cells that avoid species difference was required to demonstrate whether NTCP was the factor that contributes to the development of INH-induced hepatotoxicity. In the present study, we aimed to investigate the function of NTCP in INH-induced apoptosis using HepG2 cells, which further verified the previous conclusions in vivo and provided a basis for future research. Here, we hypothesize that the expression of NTCP is involved in the apoptosis of INH treated HepG2 cells, which is regulated by HNF4α and PGC1α, both are the known regulators of Ntcp expression and bile acid biosynthesis [16, 17]. What’s more, we specifically inhibited NTCP expression and evaluated a series of apoptotic events induced by INH.

Material and methods

Drugs and reagents

HepG2 cell line was obtained from Shanghai cell bank of Chinese Academy of Sciences. INH (Lot No. MKBV9475V, purity ≥99%) was purchased from Sigma Aldrich Co. (St. Louis, USA); Rabbit anti-SLC10A1 (NTCP/Ntcp, Biosynthesis Biotechnology, Beijing, China); rabbit anti-HNF4α/PGC1α (Abcam, Shanghai, China); rabbit anti-GAPDH (Goodhere Biotechnology, Hangzhou, China); rabbit anti-β-actin, Annexin V-FITC apoptosis detection kit and JC-1 probe (Beytime Biotechnology, Shanghai, China); RAPI 1640 (Gibco, China), Fetal Bovine Serum (Cellmax, China).

Cell culture

HepG2 cells were cultured in RAPI 1640 with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in a humidified incubator at 37°C and 5% CO₂. Passages 3-10 were used for the subsequent experiments. After adhered for 24 h, cells seeded in 96-well culture dishes at the density of 5 × 10³ cells/well were used for MTT test and 1 × 10⁵ cells/well were seeded in 6-well culture plates for all other tests. Five different test concentrations of INH (6.5, 13, 26, 52, and 104 mM) were selected for the present research [18]. All experiments were performed at least in triplicate and repeated at least three times.

Western blot analysis

The proteins were extracted as previous reports and the concentration of that was examined by the Bradford Protein Assay Kit (Beytime Biotechnology, Shanghai, China) [19]. Next proteins were electrophoresed using 8-12% SDS-PAGE and transferred onto the PVDF membranes followed by incubating in a blocking buffer (Tris 20 mM, pH 7.6, NaCl 150 mM, and Tween 20 0.1%) containing 5% nonfat milk for 1 h. At last, conjugation with primary antibodies at 4°C overnight, secondary antibodies were incubated for 1 h. ECL reagents were applied to detect the blots.

Immunocytochemistry

Cells were first washed three times with PBS, then immobilized with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 before blocked with 3% normal bovine serum. Next, antibody specific to NTCP (1:100) was incubated at 4°C overnight, and FITC-conjugated secondary antibody diluted with PBS was added in a dark place incubated for 1 h. Last, we used fluorescence microscope (Olympus) to detect and record positive staining.

MTT

HepG2 cells were subjected to growth arrest for 24 h before experimental treatment. At the end of experimental treatment, cells were incubated with 0.5% Triton X-100 before blocked with 3% normal bovine serum. Next, antibody specific to NTCP (1:100) was incubated at 4°C overnight, and FITC-conjugated secondary antibody diluted with PBS was added in a dark place incubated for 1 h. Last, we used fluorescence microscope (Olympus) to detect and record positive staining.

Flow cytometry

The percentages of apoptotic cells were measured by Annexin V-FITC apoptosis detection kit. In short, after exposed to the required experimental conditions, cells were digested with
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Trypsin and collected by centrifugation. Then cells were resuspended and fixed by 210 μL binding buffer (195 μL Annexin V-FITC, 5 μL Annexin FITC and 10 μL PI) for 15 min at room temperature away from the light. The ratios of apoptotic cells were analyzed by a flow-cyrometer.

Mitochondrial membrane potential assay

Mitochondrial depolarization was tested with JC-1 probe. Firstly, after incubated with JC-1 staining solution (5 g/mL) at 37°C for 20 min, cells in 6-well plates were washed with PBS for two times. Then cells were observed under a fluorescence microscope (Nikon) at 488 nm for green and red fluorescence. Captured images were analyzed to measure the fluorescence intensity. The relative ratio of green/red fluorescence intensity indicated mitochondrial membrane potential, and an augment in this ratio signified mitochondrial depolarization.

Statistics

All the data were expressed as a mean ± standard derivation (SD). The difference between groups analysis was performed with one-way ANOVA followed by Dunnett's test or Student's t-test. Differences were considered to be statistically significant at p<0.05.

Results

Cell viability assay

The effect of INH on cell viability was tested by MTT. HepG2 cells were treated with 6.5, 13, 26, 52 and 104 mM INH while controls were treated with RAPI 1640 medium. It was observed that cell viability decreased strikingly in a concentration-dependent manner (Figure 1).

NTCP expression was mediated by isoniazid

In order to explore the relationship between Ntcp expression and decrease of INH-induced cell viability, western blot and immunofluorescence analysis were used to identify the effect of INH on protein expression of NTCP. The result showed that NTCP expression was evoked significantly at 6.5, 13, 26 and 52 mM INH, and returned to normal level when INH rose to 104 mM (Figure 2A). Besides, Figure 2B indicated that Green fluorescence was significantly enhanced at 6.5, 13, 26 and 52 mM and weakened at 104 mM, which was in accordance with the result of western blot. Green fluorescence intensity represented the expression of NTCP.

NTCP contributes to the apoptosis induced by INH

To identify what role NTCP plays in INH-induced cell apoptosis, we conducted the following experiments. Considering high expression of NTCP and low cell viability at 26 Mm INH, this concentration was used in follow-up experiments. NTCP inhibitor CsA (20 μM) was added to HepG2 cells before the treatment of INH [20]. We observed that once NTCP was inhibited by CSA, decreased cell viability under INH exposure was partly reversed (Figure 3A).

To further confirm the significance of NTCP on apoptotic effects, apoptosis was detected biochemically by immunoblotting of whole cell lysates for the active 17-kDa cleavage fragment of caspase 3 as previously described. Caspase family play a central role in the proteolytic events of apoptosis and the expression of cleaved-caspase 3 was always taken as an indicator of cell apoptosis. Figure 3B showed that high expression of cleaved-3 was detected after treated with INH, but this effect was partly attenuated by CsA.

Flow cytometer analysis with Annexin V and PI staining was applied as another assay for apoptosis, and similar results were observed. The large population of Annexin V-positive cells (apoptotic cells) was augmented in the presence of INH, which was abolished to some extent by NTCP inhibitor CsA (Figure 3C). These findings demonstrated that NTCP was an important mediator in regulating the apoptosis induced by INH.

Figure 1. Viability of HepG2 cell was repressed by INH. INH, isoniazid. All values are represented as mean ± s.e.m. (n = 6 separated experiments, *p<0.05, ***p<0.001).
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Figure 2. INH promoted NTCP expression in HepG2 cells. A. HepG2 cells were treated with INH (6.5 mM, 13 mM, 26 mM, 52 mM, 104 mM). B. Cells were subjected to different concentrations of INH for 24 h and immunofluorescence assay of NTCP was performed. Green color indicates NTCP stained with FITC. INH, isoniazid. All values are represented as mean ± s.e.m. (n = 4 separated experiments, ***p<0.001, **p<0.01). Scale bars = 50 μm.

A

B

C

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During apoptosis, caspase activity is a contributor to mitochondrial damage. Moreover, INH has been implicated in targeting mitochondria. The damage of mitochondrial was marked by loss of mitochondrial membrane potential. Therefore, we determined mitochondrial membrane potential using JC-1 probe, which aggregates in the intact mitochondria matrix of normal cells, producing red fluorescence, and distributes widely in the impaired mitochondria of apoptotic cells, producing green fluorescence. In Figure 4A, expression of Bax protein didn’t change significantly at 26 mM INH. Interestingly, notable down-regulation of Bcl-2 protein appeared in response to INH exposure and significant decline in the Bcl-2/Bax ratio in favor of pro-apoptotic Bax protein which was partly reversed by CsA. Figure 4B showed that INH elevated the ratio of green (low potential)
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To red (high potential), indicating declined mitochondrial membrane potential and impaired mitochondria. Nevertheless, the augment of INH on mitochondrial depolarization was partly abolished by CsA. Bcl-2 is an anti-apoptotic protein on mitochondrial membrane and Bax is a pro-apoptotic protein, the two proteins participate in mitochondria-dependent apoptosis. Levels of Bcl-2 and Bax were checked by western blot in total cell lysate.

HNF4α and PGC1α participated in the regulation of NTCP by INH

To identify potential inducers of NTCP expression modulated by INH, we assessed the levels of expression of HNF4α and PGC1α. The levels of HNF4α and PGC1α were both significantly elevated after exposure to INH for 24 h, and the trend of HNF4α and PGC1α expression was in accordance with that of NTCP. The results were showed in Figure 5A, 5B.

Discussion

The highlights of this study was we demonstrate for the first time that liver transporter NTCP played a vital role in INH induced HepG2 cells apoptosis by regulating caspase activity and mitochondria function, which was closely related to its expression and perhaps mediated by HNF4α/PGC1α (Figure 6).

Previous studies about INH induced liver injury focused on oxidative stress and toxic metabolites. In the present study, we have proved NTCP was actually crucial to INH hepatotoxicity with a new perspective. As an important protein for bile homeostasis, NTCP is responsible for uptaking 80% of conjugated bile salts and approximately 50% of unconjugated bile salts from the sinusoidal blood into hepatocytes. High expression of NTCP is effective in cholestasis and may contribute to cell apoptosis and liver injury.

Due to species differences that the expression of NTCP in mouse and rat was five-fold higher than that in human, HepG2 cells were selected as target cells as the cell line being of human origin with a lot of advantages [21]. Our research showed that after treatment of 6.5, 13,
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26, 52, 104 mM INH for twenty-four hours led to significant decrease of cell viability in a concentration dependent manner (Figure 1). Caspase activity, mitochondrial membrane potential, and a major regulator of mitochondrial integrity on mitochondrial membrane, Bcl-2/Bax are reported to be regulated during the process that INH induced apoptosis. Similarly, we found increased caspase-3 activity, alleviated mitochondrial transmembrane potential and decreased ratio of Bcl-2/Bax by INH in HepG2 cells (Figures 3B, 4B). Although some reports believed NTCP is deficient in HepG2 cells, western blot and IF results in our study have shown that in HepG2 cells, NTCP is heavily evoked by INH, which was in agreement with earlier study that INH strongly increased NTCP expression in rat liver (Figure 2A, 2B). Interestingly, we found that NTCP was increased at concentration (6.5, 13, 26 and 52 mM), however, it even returned to normal level when INH rose to 104 mM, which was perhaps related to a phenomenon that liver function returns to normal despite continued treatment with the drug called ‘adaptation’ by hepatologists [22]. To further confirm NTCP was essential in INH induced HepG2 cells apoptosis, NTCP inhibitor CsA was added to cells for 3 h before INH treatment. Magically, we found that the apoptotic alterations produced by INH were partially reversed after the addition of CsA. Thus, we conclude that NTCP was an essential prerequisite of INH induced hepatotoxicity.

At last, we want to test if some signal way was involved in the regulation of INH on NTCP. Due to the knowledge that HNF4α/PGC1α were the essential regulatory factor of NTCP, we applied western blot to test the effect of INH on the expression of HNF4α/PGC1α. Interestingly, the result showed that HNF4α/PGC1α were significantly upregulated by INH, which perhaps demonstrated that the increment of NTCP was mediated by HNF4α/PGC1α (Figure 5A, 5B). Though it is well known that HNF4α/PGC1α was the regulator of NTCP, we didn’t use HNF4α/PGC1α inhibitors to further prove the effect of HNF4α on NTCP in the present study, which was the limitation of our work and need to be further proved in future.

Despite a tight correlation between NTCP and INH, as well as the critical role of NTCP during apoptosis have been clearly confirmed in our study, further work is still necessary to reveal possible mechanisms.

Conclusion

NTCP was a critical regulator in INH-induced HepG2 cells apoptosis and perhaps mediated by HNF4α/PGC1α, which facilitated a new understanding of INH-induced hepatotoxicity.

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Disclosure of conflict of interest

None.

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References

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