Proteomic analysis for the impact of hypercholesterolemia on expressions of hepatic drug transporters and metabolizing enzymes

Yan Liu*, Qiang-Hong Pu*, Ming-Jun Wu, and Chao Yu

Institute of Life Science, Chongqing Medical University, Chongqing, P.R. China

Abstract

1. Our objective is to investigate the alterations of hepatic drug transporters and metabolizing enzymes in hypercholesterolemia. Male Sprague–Dawley rats were fed high-cholesterol chows for 8 weeks to induce hypercholesterolemia. Protein levels of hepatic drug transporters and metabolizing enzymes were analyzed by iTRAQ labeling coupled with LC TRIPLE-TOF.

2. Total 239 differentially expressed proteins were identified using proteomic analysis. Among those, protein levels of hepatic drug transporters (MRP2, ABCD3, OAT2, SLC25A12, SCL38A3, SLC2A2 and SLC25A5) and metabolizing enzymes (CYP2B3, CYP2C7, CYP2C11, CYP2C13, CYP4A2 and UGT2B) were markedly reduced, but the levels of CYP2C6 and CYP2E1 were increased in hypercholesterolemia group compared to control. Decreased expressions of drug transporters MRP2 and OAT2 were further confirmed by real time quantitative PCR (RT-qPCR) and western blot.

3. Ingenuity pathway analysis revealed that these differentially expressed proteins were regulated by various signaling pathways including nuclear receptors and inflammatory cytokines. One of the nuclear receptor candidates, liver X receptor alpha (LXRα), was further validated by RT-qPCR and western blot. Additionally, LXRα agonist T0901317 rescued the reduced expressions of MRP2 and OAT2 in HepG2 cells in hypercholesterolemic serum treatment.

4. Our present results indicated that hypercholesterolemia affected the expressions of various drug transporters and metabolizing enzymes in liver via nuclear receptors pathway. Especially, decreased function of LXRα contributes to the reduced expressions of MRP2 and OAT2.

Keywords

Drug metabolizing enzymes, drug transporters, hypercholesterolemia, liver X receptor

Introduction

Drug transporters consist mainly of two super-families: ATP-binding cassette (ABC) and solute-linked carrier (SLC) transporters (Nigam, 2015). The former are the primary active drug-efflux transporters, but the latter are the primary drug-uptake transporters. Drug metabolizing enzymes (DMEs), including but not least to cytochrome P450 (CYP450) enzymes, UDP glucuronosyltransferases (UGTs), alcohol dehydrogenases (ADHs) and glutathione S-transferase (GST), are involved in biotransformation of their substrates (Sheweita, 2000). Drug transporters and metabolizing enzymes play a critical role in the disposition and govern the pharmacokinetic profiles of various substrates in the body. Therefore, induction or inhibition of drug transporters and metabolizing enzymes leads probably to clinically drug–drug or disease–drug interactions (DeGorter et al., 2012; Zhou et al., 2013). Notably, expressions and functions of drug transporters and metabolizing enzymes can be changed by many diseases. For example, in livers of obese mice fed with high-fat chow, many DMEs including CYP3A11, CYP2B10, CYP2A4, UGT1A1, SULT1A1 and SULTN were down-regulated in mRNA and protein levels (Ghose et al., 2011). Also, in type 2 diabetic rats, uptake transporter OAT2 and efflux transporters MRP2, MRP4, BCRP were increased but uptake transporter OCT2 was decreased in kidney; only efflux transporter MRP5 was reduced in liver (Nowicki et al., 2008).

Hypercholesterolemia, an elevation of total cholesterol and/or low density lipoprotein (LDL)-cholesterol in the blood, is a very common disease that affects up to 12.9% of US adults aged 20 years (Carroll et al., 2013). Hypercholesterolemia is a leading risk factor in the
development of atherosclerotic diseases, for example, coronary heart disease (CHD) (Khot et al., 2003). Hypercholesterolemia was reported to promote gene expression of ABCB1, ABCC2 (MRP2), ABCG2, SLC22A1 and SLC02B1 in peripheral blood mononuclear cells (Rodrigues et al., 2009). However, little is still known about influence of hypercholesterolemia on the expression and function of hepatic drug transporters and metabolizing enzymes. To clarify the alteration of hepatic DMEs and transporters in hypercholesterolemic condition, we conducted an investigation incorporating advanced proteomic technologies and bioinformatic analysis to explore potential candidates. Selected candidates were further validated by real time quantitative PCR (RT-qPCR) and western blot. Finally, signal pathways regulating the expression of DMEs and transporters were explored by ingenuity pathway analysis, and validated by RT-qPCR, western blot and in vitro experiments.

Materials and methods

Animals’ treatment

Adult male Sprague–Dawley rats (230–250 g) were purchased from the animal center of Chongqing Medical University. Rats were housed in a temperature-controlled room, with a 12:12-h light–dark cycle, and free access to rodent chow and water. After 1 week of acclimation period, rats were randomly assigned into two groups (n = 10/group): control and hypercholesterolemia. Rats in control group were administered to high-cholesterol chow (2.5% cholesterol). After 8-week feeding, animals were sacrificed by cervical dislocation. Livers were excised from the animal center of Chongqing Medical University. Animals were anesthetized using diethyl ether to collect whole blood, and plasma was prepared through rat sera of control and hypercholesterolemia, respectively.

Cell culture and treatment

HepG2 cells were cultured in Dulbecco’s modified Eagle’s media (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO2 and 95% O2 at 37°C. HepG2 cells were grown to sub-confluence in 75-cm2 flasks and washed twice with PBS prior to the treatment with normal serum (NS, 1%), hypercholesterolemic serum (HCS, 1%) or hypercholesterolemic serum (HCS, 1%) in combination with liver X receptor alpha (LXRα) agonist T0901317 (10 μM) in fresh medium for 24 h. Normal and hypercholesterolemic sera were prepared through rat sera of control and hypercholesterolemia, respectively.

Proteomic analysis

Proteomic analysis was performed as described by our previous study (Pu et al., 2015). Briefly, rat live tissue was homogenized in lysis buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 5% DNaSe (Promega, Fitchburg, WI), 5% RNase A (Promega), and 5% protease inhibitor cocktail set III (Merck, Darmstadt, Germany). The resulting homogenates were lysed for 30 min, freeze–thawed three times at −80°C and then intermittently sonicated for 30 s. The resulting lysate was centrifuged at 12 000 rpm for 1 h at 4°C, and the supernatant was collected. Protein concentrations were determined using the Bradford method.

iTRAQ labeling was subsequently performed according to the manufacturer’s protocol (iTRAQ 8 plex reagents, AB Sciex, Framingham, MA). Samples were precipitated with cold acetone for 2 h at −20°C, centrifuged at 3000g for 5 min, air-dried, and dissolved in dissolution buffer UA (8 M urea, 0.1 M Tris–HCl, pH 8.5). Following protein quantification using the Bradford method, 150 μg of protein per sample was reduced, alkylated, digested with trypsin for 12 h at 37°C, and labeled with two different isobaric tagging reagents. Control samples were labeled with tag 113, while hypercholesterolemia samples were labeled with tag 117. iTRAQ-labeled peptides were pooled and diluted in 100 μl strong cation exchange (SCX) buffer A [20 mM ammonium formate (v/v), pH 10.0]. After being well mixed, the samples were centrifuged at 12 000 rpm for 5 min. For peptide fractionation, iTRAQ-labeled peptides were separated using HPLC 2010A (Shimadzu, Kyoto, Japan) with a Gemini-NX 4.6 mm × 250 mm, 5 μm, 110 Å (Phenomenex, Torrance, CA; PN: 00G-4454-E0) at 0.8 ml/min and 40°C. The column was eluted with a linear gradient of 0–20% SCX buffer A (buffer A containing 20 mM ammonium formate) for 60 min, followed by a gradient of 20–100% SCX buffer B (80% acetonitrile) for 60 min. The fractions were collected at 1-min intervals, lyophilized in a vacuum concentrator and resuspended in reverse phase buffer A (98% H2O, 2% acetonitrile, 0.1% formic acid), followed by reverse-phase LC-TRIPLE TOF analysis.

For LC–MS/MS analysis, iTRAQ-labeled samples were delivered into the reverse-phase nanoLC-TRIPLE TOF 5600 instrument. Peptides were captured on a 350 μm × 0.5 mm, 3 μm Chrom XP C18 column, 120 Å (Eksigent, Dublin, CA). Peptides were then separated using a 60-min linear gradient of buffer A (0.1% formic acid in 2% acetonitrile) to buffer B (0.1% formic acid in 98% acetonitrile) at 300 nl/min. Positive ions were generated by electrospray (ion spray voltage of 2.3 kV), and quadrupole time-of-flight mass spectrometry (Q-TOF-MS) was operated in information-dependent acquisition (IDA) mode, with a scan range of 350–1500 m/z and accumulation time of 0.5 s. Peptides were sequentially selected for MS/MS analyses.

Proteomic data processing and analysis

Protein identification and quantification were performed using ProteinPilot version 4.5 (AB Sciex) against the UniProt Rattus norvegicus protein database. For false discovery rate (FDR) analysis, the data were estimated using automatic decoy searching. The relative protein expression in the hypercholesterolemia was calculated in relation to the control. Proteins were selected for further analysis if they met the following criteria: (1) expression level was significantly changed (fold change ≥2-fold versus control, p < 0.05); (2) a minimum of two peptides with 95% confidence were matched and (3) 1% global FDR was required.

For bioinformatic analyses, Gene ontology (GO) functional classification was performed using UniProt software.
Real time quantitative PCR

Total RNA was isolated from rat liver tissues using Trizol reagent (Takara, Dalian, China). cDNA synthesis was performed using PrimeScript™ RT reagent kit (Takara, Shiga, Japan). The mRNA expression of MRP2, OAT2, LXRα, FXR, PXR, CAR, PPARG and β-actin was determined by real time quantitative PCR with the following primer sets (Table 1). β-Actin was used as an internal control for RT-qPCR.

Western blot

Liver tissues or HepG2 cells were lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 0.05 mM EDTA. The resulting lysates were incubated on ice for 30 min with vortexing every 5 min. Then, the lysates were centrifuged at 12 000g for 15 min at 4°C. The supernatant was collected, and protein concentrations were determined using the BCA method. Equal amounts of protein were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat milk dissolved in TBST for 1 h at room temperature, prior to incubation with a diluted solution of the primary antibodies against MRP2 (1:50, Abcam, Cambridge, UK), OAT2 (1:500, Abcam), LXRα (1:1000, Sangon, Shanghai, China), β-actin (1:1000, CST, Danvers, MA). After being washed with TBST, membranes were probed with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were subsequently washed with TBST and incubated with enhanced chemiluminescence (ECL) reagents (Millipore, Bedford, MA). Protein bands were analyzed using the ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA).

Statistical analyses

Data are expressed as mean ± SD of at least three independent experiments. Statistical significance between two group means was assessed by Student’s t-test, and that of more than two groups was done using ANOVA, followed by with Dunnett’s t test. p < 0.05 was considered statistically significant, and all statistical analyses were carried out using Graphpad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

Results

Induction of hypercholesterolemic rat model

To induce hypercholesterolemic rat model, male Sprague-Dawley rats were fed high-cholesterol chow for 8 weeks. As illustrated in Table 2, we observed that total plasma cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) concentrations were profoundly elevated in rats fed the high-cholesterol chow (p < 0.05). Similarly, high density lipoprotein cholesterol (HDL-C) concentrations were markedly elevated in the rats fed the high-cholesterol chow, compared with that of the rat fed the standard chows. However, triglyceride (TG) concentrations were similar between the two groups. These results indicated that hypercholesterolemic rat model was successfully established. In addition, animals in hypercholesterolemia exhibited the same levels of blood glucose, AST, ALT as in control. However, animals in hypercholesterolemia exhibited higher total bile acid, direct bilirubin, creatinine, uric acid, urea than those in control (p < 0.05).

Identification of differentially expressed proteins

To explore differentially expressed proteins between hypercholesterolemia and control, proteomic analysis of iTRAQ coupled with LC TRIPLE-TOF was performed in this study. Total 2245 proteins were identified with 1% FDR from 25 606 distinct peptides derived from 71 508 spectra. With the

| Characteristics | Control (n=9) | Hypercholesterolemia (n=10) |
|-----------------|--------------|------------------------------|
| TC              | 1.6 ± 0.2    | 3.6 ± 0.8[^e]               |
| LDL-C           | 0.4 ± 0.06   | 1.3 ± 0.2[^t]               |
| HDL-C           | 1.0 ± 0.1    | 1.9 ± 0.3[^t]               |
| TG              | 1.4 ± 0.2    | 1.3 ± 0.3                   |
| blood glucose   | 6.3 ± 0.4    | 6.4 ± 0.7                   |
| ALT             | 47.7 ± 2.7   | 48.9 ± 7.3                  |
| AST             | 84.8 ± 12.9  | 88.3 ± 15.4                 |
| direct bilirubin| 0.7 ± 0.2    | 2.2 ± 0.5[^t]               |
| creatinine      | 55.2 ± 9.8   | 73.8 ± 17.4[^t]             |
| uric acid       | 205.6 ± 51.6 | 273.7 ± 71.5[^t]           |
| urea            | 5.5 ± 1.0    | 6.7 ± 1.3[^t]               |

[^e] TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride; ALT, alanine aminotransferase; AST, aspartate aminotransferase. #p < 0.05 versus control.

Table 1. Primer sequences.

| Gene       | Forward primer | Reverse primer  |
|------------|----------------|-----------------|
| Rat β-actin| CACCCGCAGTACCAACCTTC | CCCATACCCACCTACCA
| Rat MRP2   | CAATCCTGGTGTCTGTGGTC | TTTGAGGCGAGTTTCAGGAGGT |
| Rat OAT2   | GCAGCCTCTACTAATCTACATC | CATCCACCTCAAACCTACCT |
| Rat LXRα   | AGGCTCCATTCAGAAGCAAGT | CGGAGAAACAGCTCAGGCT |
| Rat FXR    | AAGTGACCTTCCAGGACCAAG | TGGCAACTCTCGTGTGC |
| Rat PXR    | CAGCCGAGCCCCAGATGTC | ATTTGCCAACACAGAGATGC |
| Rat CAR    | CATTTCCATGCTGCTGCTTGG | AGGCTGGACATCGGCTG |
| Rat PPARG  | CGGTGGATTTCTCCAGCATT | CGGACGAGGCTCATTTG |

MRP2, multidrug resistance-associated protein 2; OAT2, organic anion transporter 2; LXRα, liver X receptor alpha; FXR, farnesoid X receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; PPARG, peroxisome proliferator activated receptor gamma.
criteria of 2-fold change of expression cutoff value, 239 proteins, among in 2245 proteins, showed a significant changes ($p<0.05$) in expression level in hypercholesterolemia, compared to control (Supplementary Table 1). Of differentially expressed proteins, 188 proteins expression were up-regulated, while 51 proteins expression were down-regulated in hypercholesterolemia.

**GO of differentially expressed proteins**

To reveal the biological significance of 239 differentially modified proteins, the proteins were subject to GO analysis in the web Uniprot (http://www.uniprot.org). According to molecular function, they fall mainly into five categories: binding (64.8%), catalytic activity (56.5%), transporter activity (9.2%), structural molecule activity (5.8%) and electron carrier activity (5.4%) (Figure 1A). According to biological process, they fall mainly into cellular process (77.4%), metabolic process (77.0%), single-organism process (75.3%), biological regulation (38.1%) and response to stimulus (31.4%) (Figure 1B). GO analyses indicated that hypercholesterolemia probably affected the function of DMEs and transporters.

Figure 1. GO classification of differentially expressed proteins. (A) Molecular function. (B) Biological process. GO classification was performed using UniProt software (http://www.uniprot.org/).

**Figure 2. Influence of hypercholesterolemia on drug transporters and metabolizing enzymes.** (A) Drug transporters and (B) drug metabolizing enzymes. Differentially expressed drug transporters and metabolizing enzymes in livers were identified via molecular function analysis, which were up-regulated (≥2-fold) or down-regulated (≤0.5-fold) compared with control.

**Changed expressions of hepatic drug transporters and metabolizing enzymes in the hypercholesterolemic rats**

As expected, members of the ABC and SLC transporters, CYP450 enzymes, UGTs were present in data sets. The expression levels of drug transporters were also reduced in the hypercholesterolemic rats compared to control rats, for example ABCC2 (multidrug resistance-associated protein 2, MRP2), ABCD3, SLC22A7 (organic anion transporter 2, OAT2), SLC25A12, SLC38A3, SLC2A2 (glucose transporter 2, GLUT2) and SLC25A5 (Figure 2A). For DMEs, relative to the control rats, the expression levels of key enzymes including the CYP450 enzymes CYP2B3, CYP2C7, CYP2C11, CYP2C13, CYP4A2, and phase II enzyme UGT2B were down-regulated in livers of hypercholesterolemic rats, whereas the expression levels of CYP2C6 and CYP2E1 were up-regulated (Figure 2B).

**Validation of selected candidates MRP2 and OAT2 identified by iTRAQ**

For quantitative validation of MRP2 and OAT2, we performed RT-qPCR and western blot analysis. As shown in Figure 3(A) and (B), compared to those of control rats,
mRNA levels of hepatic MRP2 and OAT2 were remarkably decreased in the hypercholesterolemic rats \( (p < 0.05) \). Similarly, protein levels of MRP2 and OAT2 were markedly reduced in the hypercholesterolemic livers \( (p < 0.05) \) (Figure 3C and D). These results indicated that hypercholesterolemia down-regulated the expression of drug transporters MRP2 and OAT2 via suppression of gene transcription.

**Canonical signaling pathways of MRP2 and OAT2**

To highlight signaling pathways regulating expression of MRP2 and OAT2, 239 differentially expressed proteins were analyzed with a web-based tool IPA. Several canonical signaling pathways were identified, including LPS/IL-1 mediated inhibition of RXR function, NRF2-mediated oxidative stress response, xenobiotic metabolism signaling, PXR/RXR activation, FXR/RXR activation and hepatic cholestasis (Table 3). Interestingly, several nuclear receptors were identified from the analysis, which suggested that nuclear receptors signaling pathways may play a critical role in the reduced expression of MRP2 and OAT2 in hypercholesterolemia condition.

**Nuclear receptor LXR\(\alpha\) mediated the reduced expression of drug transporters MRP2 and OAT2 in hypercholesterolemia**

To clarify which nuclear receptors play a critical role in the reduced expression of MRP2 and OAT2 by hypercholesterolemia, common nuclear receptors including FXR, PXR, LXR\(\alpha\), CAR and PPAR\(\gamma\) were examined using RT-qPCR. As shown in Figure 4, mRNA level of LXR\(\alpha\) was markedly decreased but mRNA level of CAR was markedly increased in hypercholesterolemia \( (p < 0.05) \), compared to control. mRNA expressions of FXR, PXR and PPAR\(\gamma\) remained similar between hypercholesterolemia and control. Next, we examined the influence of hypercholesterolemia on LXR\(\alpha\) protein expression in livers. As shown in Figure 5, protein level of

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Table 3. IPA-defined canonical pathways of MRP2 and OAT2.

| Ingenuity canonical pathways                                      | \(-\log(p\ value)\) | Molecules                                           |
|-----------------------------------------------------------------|----------------------|-----------------------------------------------------|
| LPS/IL-1 mediated inhibition of RXR function                    | 4.4                  | ABCC2, FABP1, Cyp4a14, ALDH1A1, CAT, CYP2C8, CYP2C19, ALDH1B1, FMO3, CYP2C9, ALDH4A1 |
| NRF2-mediated oxidative stress response                         | 4.4                  | SOD1, ABCC2, CAT, ERP29, EPHX1, PPB, VCP, NQO1, PRDX1, FTH1 |
| Xenobiotic metabolism signaling                                  | 4.2                  | ABCC2, ALDH1A1, CAT, CYP2C8, UGT2B28, CYP2C19, ALDH1B1, CES1, NQO1, FMO3, CYP2C9, ALDH4A1 |
| PXR/RXR activation                                              | 4.0                  | ABCC2, ALDH1A1, CYP2C8, G6PC, CYP2C19, CYP2C9 |
| FXR/RXR activation                                              | 2.5                  | ABCC2, CLU, SLC22A7, G6PC, FGA, TTR                  |
| Hepatic cholestasis                                             | 0.3                  | ABCC2, SLC22A7                                       |

Differentially expressed proteins were analyzed with IPA software, and then canonical signaling pathways relating MRP2 (ABCC2) and OAT2 (SLC22A7) were identified. RXR, retinoid X receptor; NRF2, nuclear factor E2-related factor 2; PXR, pregnane X receptor; FXR, farnesoid X receptor.
LXRα in hypercholesterolemia was also significantly decreased compared to control (p < 0.05). The results indicated that nuclear receptors LXRα and/or CAR regulated expression of MRP2 and OAT2 in hypercholesterolemic livers.

To confirm that interference of LXRα function mediated the reduced expression of drug transporters MRP2 and OAT2 in hypercholesterolemia, we examined the effect of LXRα agonist T0901317 on LXRα, MRP2 and OAT2 in the presence of hypercholesterolemic serum in HepG2 cells. As illustrated in Figure 6, we found that compared to those in NS treatment, protein levels of LXRα, MRP2 and OAT2 were reduced in hypercholesterolemic serum treatment (p < 0.05). However, LXRα agonist T0901317 (10 μM) rescued the reduced protein levels of LXRα, MRP2 and OAT2 in hypercholesterolemic serum treatment (p < 0.05). These results clearly indicated that the decreased expressions of
MRP2 and OAT2 by hypercholesterolemia are, at least partly, nuclear receptor LXRα-dependent.

**Discussion**

In this study, using a comparative proteomic analysis, we found that hypercholesterolemia affected the expression of hepatic drug transporters and metabolizing enzymes in the high-cholesterol chow induced rat model of hypercholesterolemia. Specifically, we found that protein levels of DMEs including CYP2B3, CYP2C7, CYP2C11, CYP2C13, CYP4A2 as well as UGT2B were markedly reduced, while CYP2E1 and CYP2C6 were markedly increased in the livers of hypercholesterolemic rats. Additionally, we observed that protein levels of various drug transporters, especially MRP2 and OAT2, were also reduced in livers of hypercholesterolemic rats.

Multidrug resistance-associated protein 2 (MRP2, ABCC2), named canalicular multispecific organic anion transporter (cMOAT) or canalicular multidrug resistance protein (cMRP), is an important efflux pump at hepatocyte canalicular membrane. MRP2 mediates hepato-biliary excretion of a variety of endogenous and xenobiotic compounds, including bilirubin glucuronide, anticancer drug, antihypertensive agents and antibiotics (Jedlitschky et al., 2006; Kosaka et al., 2015; Marquez & Van Bambeke, 2011; Paulusma & Oude Elferink, 1997). By contrast, organic anion transporter 2 (OAT2, SLC22A7), named novel liver-specific transporter (NLT), is an important uptake pump at hepatocyte sinusoidal membrane and renal basolateral membrane of proximal tubule. OAT2 also mediates hepatic and renal elimination of endogenous and xenobiotic compounds including creatinine (Paulusma & Oude Elferink, 1997), uric acid (Sato et al., 2010), statins, diuretics, anticancer drug, antibiotics (Burckhardt, 2012; Rizwan & Burckhardt, 2007). Therefore, MRP2 and OAT2 are considered to be the main membrane transporters governing the pharmacokinetic profile of drugs. In this study, we found that hypercholesterolemia reduced the gene expressions of MRP2 and OAT2 in the livers of rats, which suggested that the transport activities of MRP2 and OAT2 were inhibited by hypercholesterolemia. Interestingly, we indeed observed that MRP2 substrate direct bilirubin (conjugated bilirubin) and OAT2 substrates creatinine as well as uric acid were significantly elevated in hypercholesterolemic serum. Of course, creatinine and uric acid were also eliminated through other renal transporters, so the transporters mediating the renal
excretion of creatinine and uric acid need to be investigated in the future study.

To elucidate the signaling pathways that regulate the expression of drug transporter and metabolizing enzymes genes, IPA was performed to analyze 239 differentially modified proteins. The identified canonical pathways included many nuclear receptors, such as PXR and FXR. We hence examined mRNA levels of common nuclear receptors using RT-qPCR, and observed that only LXRα mRNA level was repressed and CAR mRNA level was induced, but PXR, FXR and PPARG mRNA level remained unchanged in hypercholesterolemic livers. Also, western blot confirmed that LXRα protein level was decreased in hypercholesterolemic livers. Previous studies reported that LXRα agonist T0901317 activated transcription of MRP2 (Chisaki et al., 2009). Therefore, we speculated that reduced MRP2 expression, even OAT2, may be caused by interference of LXRα signal. As expected, LXRα agonist can rescue the hypercholesterolemic serum-repressed MRP2 and OAT2 protein levels in HepG2 cells. Besides LXRα, the role of another nuclear receptor CAR in regulating MRP2 and OAT2 needs to be considered because MRP2 and OAT2 have been shown to be regulated by CAR. Interestingly, the CAR activator phenobarbital down-regulated OAT2 but up-regulated MRP2 expression (Jigorel et al., 2006; Kast et al., 2002; Wagner et al., 2005). Taken together, we suggest that hypercholesterolemia decreased OAT2 expression via LXRα and CAR pathways, while decreased MRP2 expression only via LXRα pathway.

In conclusion, using a comparative proteomic analysis we found 239 differentially expressed proteins in hypercholesterolemic livers, among which a variety of drug transporters and metabolizing enzymes were identified, especially two key transporters MRP2 and OAT2. Also, we deciphered the detailed molecular mechanisms by which hypercholesterolemia decreased MRP2 and OAT2 expressions and activities, and that dysfunction of two nuclear receptors LXRα and CAR are responsible for the lower expressions of MRP2 and OAT2. Additionally, our results provide a rationale to monitor the effectiveness and toxicity of drug transporters and metabolizing enzymes-related drug therapy in hypercholesterolemic individuals.

Declaration of interest
The authors have declared no conflict of interest.

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Supplementary material available online
Supplementary Table 1