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Research Article

Hyperlipidemia: Insights into Mechanisms Involved in Modulation of Drug Pharmacokinetics and Response

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Abstract. Hyperlipidemia is associated with disturbances in plasma lipoproteins. These changes were shown to modify the pharmacokinetics of some lipophilic lipoprotein-bound drugs with some of these changes being clinically significant. In this manuscript potential mechanisms that might be involved in hyperlipidemia-induced changes in drug pharmacokinetics such as modulation of p-glycoproteins and liver metabolising enzymes activities and/or expression were investigated. P-glycoprotein gene expression (MDR1A/B) and activity was assessed in porcine (LLC-PK1) and murine (NRK-52E) proximal tubular renal epithelial cells that were maintained in normolipidemic- or hyperlipidemic-serum containing media and was compared to expression levels in serum-deprived cells. Gene expression of low density lipoprotein receptor (LDLr) and MDR1A/B along with cytochrome P450 CYP3A2 and 2C11 were also investigated in hepatocytes from normolipidemic and hyperlipidemic rats using qRT-PCR. Hyperlipidemic serum did not affect MDR1A/B gene expression in LLC-PK1 and NRK-52E cells. However, it significantly reduced p-glycoprotein activity in rifampin-treated and non-treated LLC-PK1 cells. Interestingly, p-glycoprein gene expression was induced in LLC-PK1 and NRK-52E cell lines upon serum starvation. Moreover, hepatocytes from hyperlipidemic rat livers had reduced LDLr, MDR1A/B, and CYP2C11 mRNA levels compared to normolipidemic rats. It is concluded that hyperlipidemia-induced changes in drug pharmacokinetics might be attributed to modulation of drug efflux/uptake, metabolism and/or excretion. Further, p-glycoprotein gene expression is potentially inducible by serum starvation in renal proximal tubular cells, which might be a novel detoxification mechanism by which these cells adapt to stressful conditions.

Keywords: hyperlipidemia, p-glycoprotein, cytochrome P450, kidney, liver, pharmacokinetics

1. Introduction

Hyperlipidemia is a disease state associated with disturbances in plasma lipoprotein levels. Since lipophilic drugs such as amiodarone and halofantrine bind to plasma lipoproteins, hyperlipidemia expectedly was shown to alter their pharmacokinetic profiles [1–4]. Interestingly though, hyperlipidemia-induced changes in drug pharmacokinetics is not limited to lipophilic drugs, there is an old report that shows it also changes the pharmacokinetics of hydrophilic drugs such as atenolol thus extending its effects on drug pharmacokinetics beyond those expected based on simple protein-binding interactions [5].

Pharmacokinetics alterations that are commonly produced by hyperlipidemia include decreased drug clearance and volume of distribution. These changes have been observed for several drugs including amiodarone and dronedarone [2, 6]. Stereo-selective changes in the clearance and volume of distribution of ketoconazole were also observed in response to experimental hyperlipidemia where an increase in steady state volume of distribution and a decrease in liver uptake were observed for the (-) enantiomer [7]. Tissue-specific

changes in cyclosporine uptake were also observed, where increased levels were observed in blood, kidney, and liver and decreased levels were observed in heart and spleen of hyperlipidemic rats compared to normolipidemic controls [8].

Indeed, some of these pharmacokinetics changes were significant enough to cause modifications in drug response and/or toxicity. For example, increased uptake of amiodarone in the heart tissue of hyperlipidemic rats was associated with enhanced QTc and PR interval prolongation with cholesterol to triglycerides plasma ratio being linearly related to the increases noted in both QTc and PR prolongation and amiodarone concentrations [9]. In addition, hyperlipidemic rats treated with cyclosporine showed increased cyclosporine uptake in their kidneys after single dose administration. After repeated doses of cyclosporine (for 7 days), hyperlipidemic rats showed more histopathological defects in their kidneys than their normolipidemic counterparts [8].

Mechanisms thought to be involved in hyperlipidemia-induced changes in drug pharmacokinetics include increased plasma protein binding, downregulation of some cytochrome P450 isoforms (CYPs) and some transport proteins [10-13]. This work is aimed at exploring the effect of experimental hyperlipidemia on p-glycoprotein gene expression and activity using proximal tubular renal epithelial cell line derived from porcine and rat kidneys. Rifampin was used to induce p-glycoprotein expression in LLC-PK1 cell line as these cell lines are known to lack p-glycoprotein [14, 15]. Gene expression of some CYP isoforms that are commonly involved in drug metabolism was also assessed in rat hepatocytes under normolipidemic and hyperlipidemic conditions.

Results presented herein show that hyperlipidemic serum does not alter MDR1A/B gene expression in the kidney (LLC-PK1 or NRK-52E cell line); however it produces significant reductions in p-glycoprotein activity (in LLC-PK1) potentially through post-transcriptional modifications. Interestingly, serum deprivation produced significant upregulation of p-glycoprotein gene expression which was inhibited by high lipoprotein levels especially in NRK-52E cell line. Furthermore, hepatocytes exposed to hyperlipidemic serum showed decreased LDL receptor, MDR1A/B and CYP2C11 gene expression. Collectively, these results emphasize that hyperlipidemia can indeed alter drug pharmacokinetics by multiple mechanisms, such as modulation of drug uptake, metabolism and excretion.

2. Materials and Methods

LLC-PK1 and NRK-52E cells were purchased from American type culture collection (Manassas, VA, USA). Poloxamer 407, medium 199, fetal bovine serum, rifampin, ethylene diamine tetraacetic acid (EDTA) and rhodamine-123 were purchased from Sigma (St Louis, MO, USA). Methanol, hydrochloric acid, dimethyl sulfoxide and sodium hydroxide were obtained from Caledon labs (Georgetown, Ontario, Canada). Penicillin-streptomycin (10,000 units penicillin; 10,000 µg streptomycin/ml), Dulbecco's Modified Eagle Medium and trypsin were obtained from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA).

Hyperlipidemic serum was obtained from Sprague–Dawley rats (Charles River, Quebec, Canada) after being rendered hyperlipidemic. Hyperlipidemia was induced by intraperitoneal administration of 1 g/kg poloxamer 407 (a triblock copolymer made up of a central hydrophobic block of polypropylene glycol linked to two hydrophilic polyethylene glycol blocks, 0.13 g/ml in normal saline). To ensure proper injection of the dose, the animals were lightly anesthetized with isoflurane. Hyperlipidemic serum (HLS) was collected by cardiac puncture about 36 h after poloxamer injections whereas normal lipidemic serum (NLS) was collected from untreated rats. Protocols involving animal use were approved by the University of Alberta Health Sciences Animal Care and Use Committee where the animal and tissue culture procedures were conducted (data analysis and manuscript preparation however were done in Egypt). Rhodamine-123 stock solution (121 µM) was prepared in 0.23% methanol in autoclaved water and dilution of this stock (to yield 10 µM; final concentration) in the respective medium was used for cell treatment.

2.1. Cell culture conditions. LL-CPK1, proximal tubular renal epithelial cell line derived from porcine kidneys (passages 201-220), were grown in medium 199 (9.5 g/l) containing 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 100 unit/ml penicillin and 100 µg/ml streptomycin. NRK-52E cells, proximal tubular renal epithelial cell line derived from rat kidney (passages 19-34), were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 µg/ml streptomycin. Both cell lines were maintained in 95% O_2 and 5% CO_2 humidified incubator at 37° C and sub-cultured every five days using 0.25% trypsin/2mM EDTA in phosphate buffered saline (PBS). LL-CPK1 and NRK-52E cells were sub-cultured in 1:6 and 1:3 ratios, respectively.

Cell pellets of hepatocytes isolated from hyperlipidemic (HLC) and normolipidemic (NLC) rats that were treated with diluted (5%) normolipidemic or (5%) hyperlipidemic serum (n=3-6 per/group) were obtained from Dr. Dion Brocks, University of Alberta. These cells were used to explore the effect of hyperlipidemia on different enzymes involved in drug metabolism, p-glycoprotein and LDL receptor gene expression in the liver.

2.2. RNA extraction and cDNA synthesis. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was then synthesized by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

2.3. Quantification by real-time PCR. Quantitative analysis of mRNA expression was performed by real-time PCR by subjecting the resulting cDNA to PCR amplification in 96-well optical reaction plates using the ABI Prism 7500 System (Applied Biosystems). The 25 µl reaction mix contained 0.1 µl of 10 µM forward primer, 0.1µl of 10 µM reverse primer, 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample. Forward primer sequences were GACAGGACATCAGGACCATCAAT, CAACGGTGGCTGCCAGTAC, CACCAGCTATCAGTGGATTTGG, TACTACAAGGGCTTAGGGAG and CAAGGTCATCCATGACAACTTTG for MDR1A/B, LDL-r, CYP2C11, CYP3A2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Backward primer sequences were GACGTTTTCTCGGCCATAGC, GAACTTGGGTGAGTGGGCAC, GTCTGCCCTTTGCACAGGAA, CTTGCCTGTCTCCGCCTCTT and GGGCCATCCACAGTCTTCTG for MDR1A/B, LDL-r, CYP2C11, CYP3A2 and GAPDH, respectively. No-template controls were incorporated to test for the contamination of any assay reagents. The thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve analysis was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.4. Real-time PCR data analysis. Real-time PCR data were analyzed using the relative gene expression (i.e.,ΔΔCT) method, as described in Applied Biosystems User Bulletin No. 2 and explained by Livak and Schmittgen [16]. The data are presented as fold of change in gene expression normalized to endogenous reference gene (GAPDH) and relative to a calibrator. Control cells treated with medium only was used as the calibrator to measure the change in gene expression by different treatments.

2.5. P-glycoprotein activity measurement. For the purpose of p-glycoprotein activity measurement, LL-CPK1 and NRK-52E cells were plated at a density of approximately 1.5×10^5 cells/ml and 2.5×10^5 cells/ml, respectively in 24-well plates (0.5 ml/well). After 6 hours of culture, p-glycoprotein activity was induced in a subset of LL-CPK1 cells by replacing the medium with rifampin (25 µM) containing medium [17]; no-rifampin-treated LL-CPK1 controls were also included in our study. The treatment with rifampin was continued for one week with medium being replaced every 48 hours. In the case of NRK-52E cells, cells were exposed to HL and NL serum without pre-exposure to rifampin. One week after rifampin treatment (for LL-CPK1 cells) or one day after culture (for NRK-52E cells), both cell lines were exposed to either medium only or 20% NL or 20% HL serum containing medium. Serum treatment was continued for 24 hours then p-glycoprotein activity was assessed by measuring rhodamine-123 uptake under normal and hyperlipidemic conditions. Briefly, serum-containing medium was aspirated and cells were washed three times with PBS. Thereafter, medium containing 10 µM rhodamine-123 was added to cells. At different time points, the medium was aspirated and cells were washed three times with ice-cold PBS then cells were lysed by adding 0.5 ml of 0.3 M NaOH that is neutralized with 0.3 M HCl. Rhodamine-123 cellular levels were measured using a fluorometric assay at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.6. Statistical analysis. Unpaired student *t*-test, two-way analysis of variance, one-way analysis of variance followed by Dunn's test or Holm-Sidak test for multiple comparisons were used as appropriate to assess differences between groups. SigmaPlot 13 software was used in conducting the statistical analyses and the significance level was set at <0.05.

3. Results

MDR1A/B gene expression in NRK-52E cell lines was not altered by exposing the cells to NL or HL rat serum (Figure 1A). However, it was markedly upregulated (29-fold) in serum-deprived cells (for 24 h). Cells previously incubated with NL serum showed significantly greater extent of MDR1A/B upregulation (44-fold) upon NL serum deprivation. The extent of upregulation was significantly lower in cells previously incubated with HL serum and equivalent to cells incubated with medium only.

Upregulation of MDR1A/B gene expression was also noted in LLC-PK1 cells that were not previously-treated with rifampin upon media starvation (Figure 1B). However, it was not upregulated in LLC-PK1 cells previously incubated with NL or HL rat serum upon serum starvation (Figure 1B). Rifampin-treated LLC-PK1 cells showed almost no change in MDR1A/B gene expression when incubated with medium or NL or HL sera and also showed no change in p-glycoprotein gene expression in response to serum starvation (Figure 1C).

LDLr gene expression was significantly reduced by 49% ($p=0.002$) and 20% ($p=0.025$ Figure 2A) in normolipidemic hepatocytes which were incubated with HLS (NLC+HLS) and hepatocytes isolated from hyperlipidemic rats (HLC). MDR1A/B gene expression was reduced when normolipidemic hepatocytes were incubated with NLS (NLC+NLS) and HLS (NLC+HLS) (56% and 64%, respectively with p values being <0.001 Figure 2A). MDR1A/B gene expression was also reduced in hepatocytes isolated from hyperlipidemic rats (HLC) by 30% ($p=0.034$) compared to normolipidemic hepatocytes. Hyperlipidemia specifically reduced CYP2C11 gene expression by 44% ($p=0.006$) and 70% ($p=0.0003$ Figure 2B) in HLC and NLC+HLS groups, respectively. Unexpectedly, CYP3A2 gene expression was reduced when serum was added to hepatocyte cultures *in vitro* irrespective of its type, NL or HL with its mRNA levels

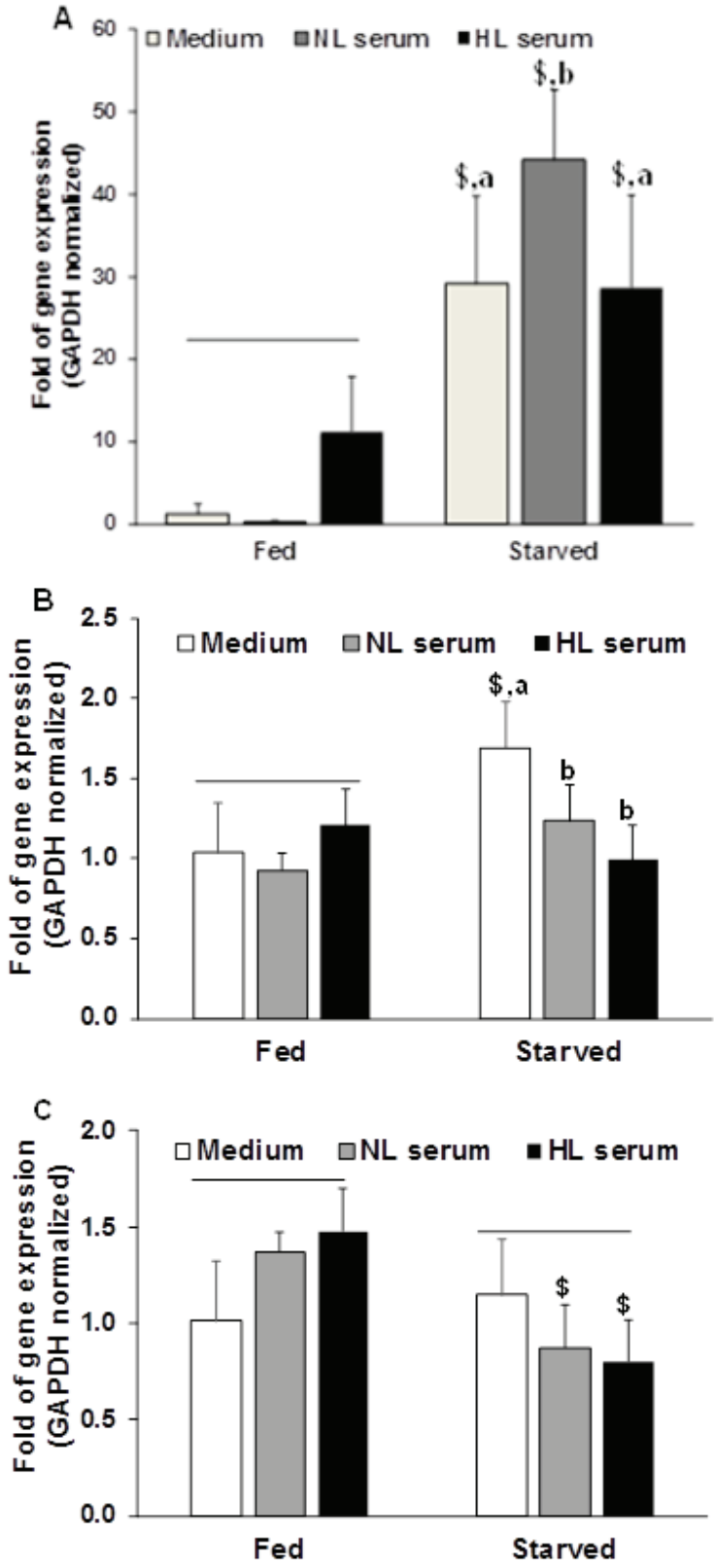

Figure 1: Fold of MDR1A/B gene expression in A) NRK-52E, B) Non-Rifampin treated LL-CPK1 and C) Rifampin-treated LL-CPK1 cells. Cells were exposed to either medium-only or 20% NL or 20% HL serum for 24 h (Fed), then serum was removed and cells were starved for 24 h (Starved). Two-way analysis of variance followed by Holm-Sidak test was used to assess differences in MDR1A/B mRNA. Groups under the same line are statistically equivalent, $ indicate significant difference from the respective serum-fed cells, and different letters indicate significant difference between different sera.

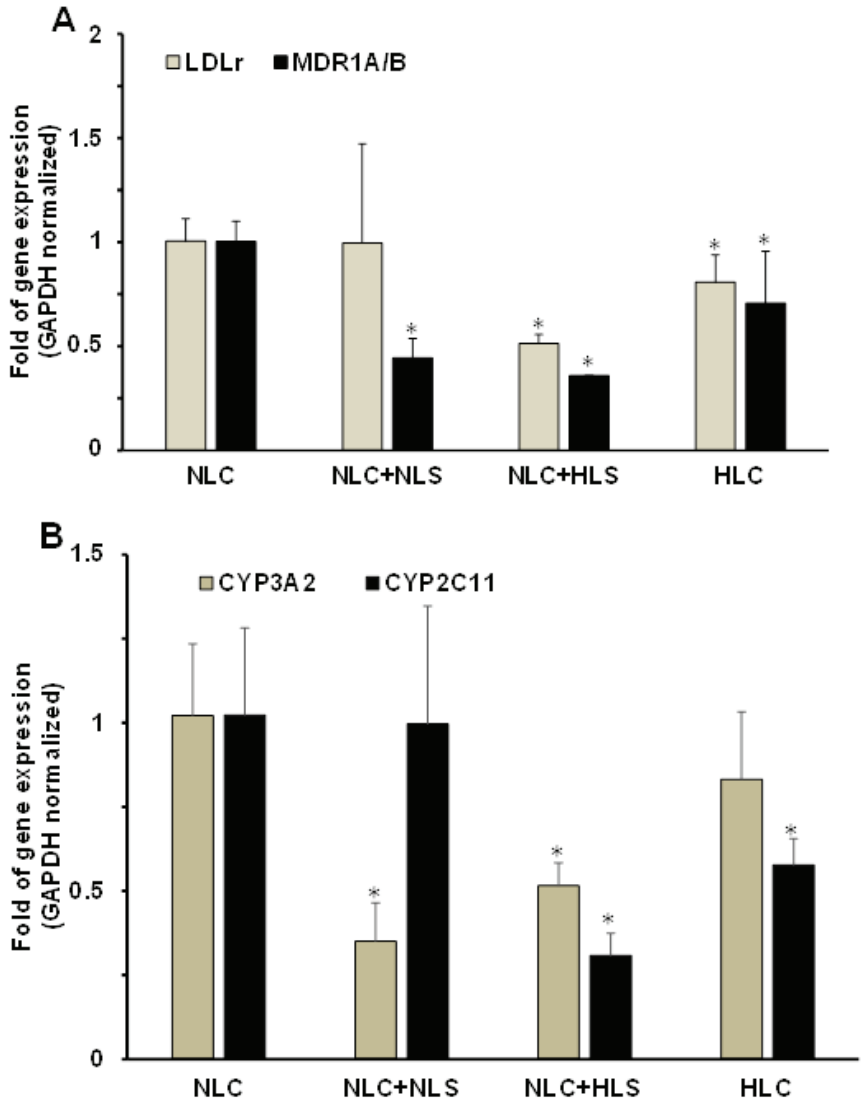

Figure 2: A) Fold of LDLr and MDR1A/B gene expression, and B) Fold of CYP3A2 and CYP2C11 gene expression in normolipidemic hepatocytes (NLC), normolipidemic hepatocytes treated with normolipidemic serum (NLC+NLS), normolipidemic hepatocytes treated with hyperlipidemic serum (NLC+HLS), and hyperlipidemic hepatocytes (HLC). One way analysis of variance followed by Dunn's test or Holm-Sidak test were used to assess differences among the groups.* indicates significant difference from NLC.

measured in hyperlipidemic hepatocytes being essentially similar to normolipidemic hepatocytes (Figure 2B).

Although rifampin did not induce MDR1A/B gene expression in LLC-PK1 cell line, it clearly induced p-glycoprotein activity as measured by rhodamine-123 uptake, potentially through post-transcriptional mechanisms (Figure 3). Average increase in p-glycoprotein activity ranged from 12-35% at the different tested time points. Additionally, hyperlipidemic serum consistently inhibited p-glycoprotein activity in LLC-PK1 cells that were previously treated or untreated with rifampin with percent of inhibition ranging from 22%-100% (Figure 4).

4. Discussion

Hyperlipidemia is associated with disturbances in plasma lipoprotein levels. These changes expectedly modify the pharmacokinetics of lipoprotein-bound drugs such as amiodarone and halofantrine. Some of these changes resulted in modifications in the response and toxicity of different drugs. For example, increased uptake of amiodarone in the heart tissue of hyperlipidemic rats was associated with enhanced QTc and PR interval prolongation [9]. In addition, hyperlipidemic rats show increased cyclosporine A uptake in their kidneys after single dose administration with more

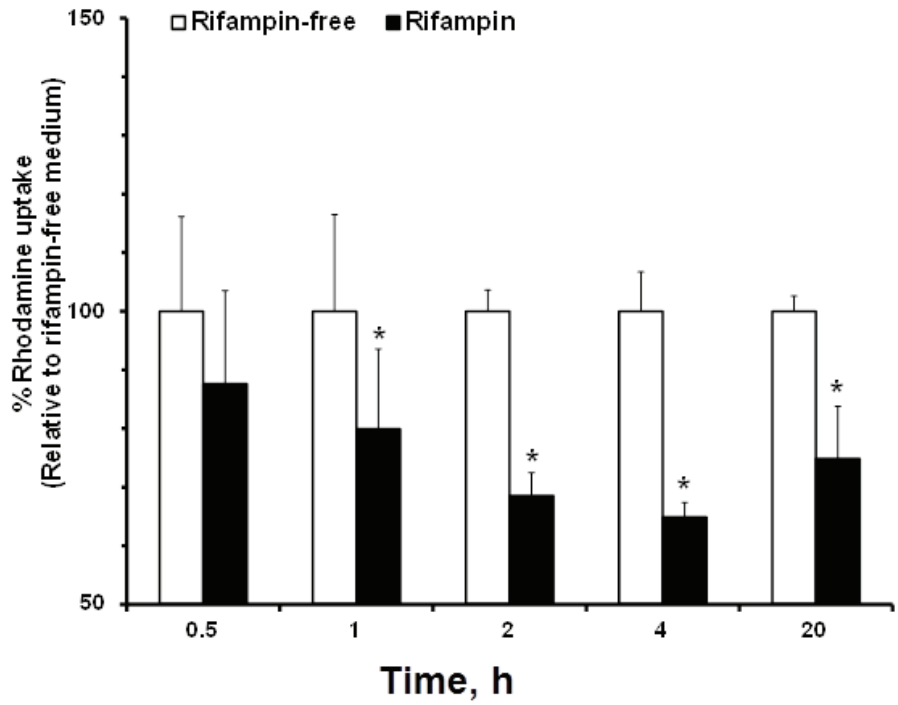

Figure 3: Rohdamine-123 uptake in rifampin treated or untreated LL-CPK1 cells. Cells were treated with 25 μM rifampin containing medium for a week. Thereafter, rohdamine-123 (10 μM) uptake was measured at different time points. Data are expressed as percent uptake relative to cells exposed to rifampin-free medium (with rifampin-free incubations being equivalent to 100%). Differences were assessed using unpaired two-tailed student *t*-test. * represent significant difference between rifampin-treated and untreated cells.

histopathological defects being detected in their kidneys upon repeated administration compared to normolipidemic rats [8]. Results presented herein confirm previous findings that hyperlipidemia can indeed alter drug pharmacokinetics by multiple mechanisms, including modulation of drug transport, metabolism and excretion.

The finding of upregulation of p-glycoprotein gene expression upon serum starvation in NRK-52E and non-rifampin treated LLC-PK1 cell lines is particularly interesting. In fact previous reports have indicated that LLC-PK1 cell line lack p-glycoproteins [14, 15] however p-glycoprotein gene expression was detectable here in this cell line. This can be attributed to the use of the sensitive real-time PCR technique in this study. It is worth mentioning that an earlier report shows that p-glycoprotein transfected NIH-MDR1-G185 maintained in glucose-deficient buffer spent significant amount of energy (>2-fold) on detoxification by p-glycoprotein relative to basal values than glucose-fed cells [18]. Also, it has been shown that p-glycoprotein half-life is increased (by 4 to 6-fold) in human and hamster multidrug-resistant cell lines when they are grown in the presence of a low serum level [19]. These observations generally support the results presented herein of overexpression of p-glycoprotein gene under conditions of serum starvation and highlight the importance of maintaining p-glycoprotein function under different stressful conditions to the cell viability.

P-407 induced-hyperlipidemia has been attributed to inhibition of lipoprotein lipase, induction of HMG-CoA reductase, and hepatic cholesterol depletion or redistribution into plasma [20-23]. The observation of reduced LDLr gene expression in hepatocytes exposed to hyperlipidemic serum or serum derived from P-407 rat model is consistent with the previously reported observation of reduced LDLr protein expression in P-407 mice [24]. According to authors of this article P-407 induced hyperlipidemia is actually mediated *via* enhanced cholesterol synthesis as indicated by upregulation of HMG-CoA reductase protein and activity coupled to reduced cholesterol internalization by the liver evidenced by the reduced LDLr protein level which is consistent with the results presented herein.

Nonspecific reduction in MDR1A/B gene expression with serum irrespective of lipid status excludes inhibitory effect of hyperlipidemic serum by itself on p-glycoprotein gene expression in the liver. However, hepatocytes isolated from *in vivo* produced rat model of hyperlipidemia had a significantly reduced MDR1A/B gene expression which indicates that an indirect inhibitory effect of hyperlipidemia, as a disease/pathology, on MDR1A/B gene expression is still likely. Further studies preferentially from post-mortem

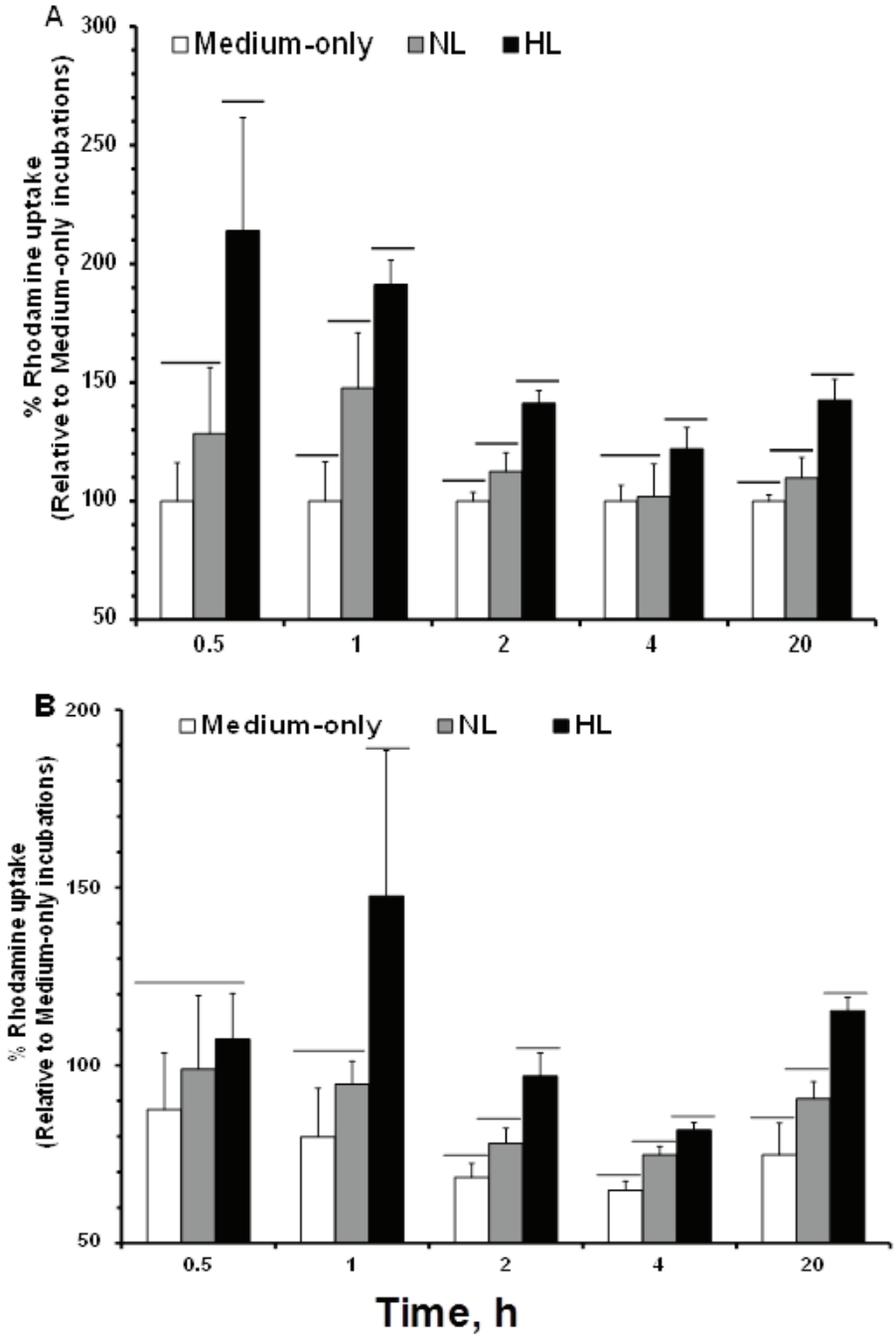

Figure 4: Rohdamine-123 uptake in rifampin-free (A) or rifampin-treated (B) LL-CPK1 cells. Cells were exposed to either medium-only or 20 % NL or 20% HL serum for 24 h and then rohdamine-123 (10 μM) uptake was measured. Data are expressed as percent uptake relative to cells exposed to rifampin-free medium-only (equivalent to 100%). One way analysis of variance followed by Dunn's test or Holm-Sidak test were used to assess differences among the groups at each time point. Breaks between horizontal lines above data bars denote significant differences from other data point(s).

human samples should be useful in drawing a solid conclusion on this matter. It is worth mentioning that More et al.2017 shows that p-glycoprotein activity of rat brain capillaries is altered by exposure to lipids. Specifically, when brain capillaries were exposed to 10–50 μM linoleic acid, p-glycoprotein transport activity was increased [25]; an effect that was abolished by the PPAR-α antagonist, GW6471. Interestingly, the authors also show that short-term fasting induced p-glycoprotein transport activity in brain capillaries *via* fasting mediated enhancement of serum non-esterified

fatty acids-PPARα activation. These results further support the renal cell line data presented herein showing marked upregulation of p-glycoprotein mRNA in response to serum starvation.

It is worth mentioning that lipoproteins are increasingly recognised as drug carriers, indeed they can associate with drugs and mediate their transport into tissues in lipid-associated forms. Clopidogrel for example accumulated in VLDL/LDL fractions in LDLr-mutant mice and was taken up by the liver when functional LDLr was expressed, suggesting that LDLr plays a key role in the uptake of VLDL/LDL-associated drugs *in vivo* [26]. Undeniably, P-glycoprotein is a major transporter involved in the hepatobiliary excretion of drugs such as digoxin and vincristine [27]. Inhibition of this efflux transporter in hyperlipidemia patients therefore could potentially impair the clearance of different drugs eliminated by this route.

It was reported earlier that a 15-day treatment with 25 μM of rifampin in the LLC-PK1 tubular renal cell line significantly induces P-glycoprotein mRNA levels [17]. Although this observation wasn't reproducible here, both data presented here and in the earlier report, show that indeed rifampin induces p- glycoprotein activity in this cell line. In addition, inhibition of p-glycoprotein activity in rifampin-treated or non-treated LLC-PK1 cell lines by exposure to hyperlipidemic serum is consistent with an earlier report which shows that hyperlipidemic serum inhibits p-glycoprotein activity in NRK-52E cell line [13]. These results suggest functional impairment of p-glycoprotein efflux transport in the kidneys of hyperlipidemic patients and therefore potential reduction of the clearance of different drugs actively secreted by this transporter.

In summary this report shows that hyperlipidemia-induced changes in drug pharmacokinetics might be attributed to modulation of drug transport, CYP-mediated drug metabolism and excretion. Additionally, p-glycoprotein gene expression is induced by serum starvation in LLC-PK1 and NRK-52E cell lines, which might be a potentially novel detoxification mechanism by which cells adapt to different stressful conditions.

Competing Interests

The author declares no competing interests.

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