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Effects of atorvastatin on *CYP3A4* and *CYP3A5* mRNA expression in mononuclear cells and CYP3A activity in hypercholeresterolemic patients

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ABSTRACT

Background: Variability of response to statins has been related to polymorphisms in genes involved in cholesterol homeostasis and statin metabolism, such as *CYP3A4* and *CYP3A5*. We investigated the effects of atorvastatin on *CYP3A4* and *CYP3A5* mRNA expression in mononuclear cells and on CYP3A activity and their interactions with common variants.

Methods: Unrelated individuals (n = 121) with hypercholesterolemia (HC) were treated with atorvastatin (10 mg/day/4 weeks). Ninety-two normolipidemic (NL) subjects were selected as a control group. Genotype analysis of *CYP3A4**1B (rs2740574), *CYP3A4**22 (rs35599367), *CYP3A5**3C (rs776746), and *CYP3A5**1D (rs15524) and mRNA levels in peripheral blood mononuclear cells (PBMCs) were estimated. CYP3A activity was phenotyped by the urinary cortisol to 6-beta-hydroxy-cortisol ratio.

Results: LDL cholesterol reduction in response to atorvastatin was positively correlated with change in *CYP3A4* ($R^2 = 0.039$, $p = 0.037$) and *CYP3A5* ($R^2 = 0.047$, $p = 0.019$) mRNA levels and negatively correlated with CYP3A activity ($R^2 = 0.071$, $p = 0.022$). *CYP3A5**3C (AGT haplotype) was associated to lower basal *CYP3A5* mRNA expression in HC ($p < 0.045$), however none of the haplotype groups impacted treatment.

Conclusion: It is likely that cholesterolemia status changes promoted by atorvastatin play a role in regulating *CYP3A4* and *CYP3A5* mRNA expression in PBMCs, as well as CYP3A activity. *CYP3A5**3C (AGT haplotype) also contributes for the variability of *CYP3A5* mRNA levels in PBMCs.

1. Introduction

Statins are reversible inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, which catalyzes the rate-limiting step in the *de novo* biosynthesis of cholesterol by acetyl-CoA [1]. Reduction of intracellular cholesterol subsequently induces the expression of the low-density lipoprotein (LDL) receptor, resulting in increased LDL uptake by hepatic and peripheral cells, thereby dramatically reducing circulating cholesterol levels [2]. Pleiotropic effects include improving endothelial function, decreasing vascular inflammation and enhancing plaque stability [3]. Statin use contributes to an approximate 30% reduction in cardiovascular disease (CVD) [4], and statins are one of the most commonly prescribed drugs worldwide.

Atorvastatin is administered orally as an active acid form and is metabolized primarily by cytochrome P450 3A4 (CYP3A4) and CYP3A5 in the gut and liver, and it is further conjugated by uridine diphosphate glucuronosyl transferases 1A1 and 1A3 [5]. The uptake and efflux membrane drug transporters expressed in liver and intestinal cells contribute to the overall bioavailability of the atorvastatin [5–7].

CYP3A4 and CYP3A5 are important drug metabolizing enzymes due to their abundance in the intestinal epithelium and liver, and their ability to metabolize a large number of chemically unrelated drugs [8]. Inter-individual variability in CYP3A4 and CYP3A5 liver expression is very high (10- to 100-fold). This variability has been suggested to be associated with variants in the genes encoding CYP3A4 and CYP3A5 and

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may contribute to the differences found in response to several drugs, including atorvastatin and other statins [9,10].

There is also great interindividual response to statin treatment. It is thought that genetics may play an important role in the biodisposition and safety of statins [11]. Understanding and addressing the pharmacogenomic variability of statins has been challenging and numerous efforts have been undertaken to discover a surrogate marker for statin response to treatment.

CYP3A4 and CYP3A5 expression and activity in the liver are major determinants of drug efficacy, toxicity, and hence, therapeutic outcome [10]. Hepatocytes are the main site of action for statins. Increased *CYP3A* mRNA expression and CYP3A protein levels in hepatocytes have been demonstrated to be increased by statins [12], ranging from 6 to 20 fold the basal level [13,14]. Moreover, *CYP3A4* mRNA expression was increased when HepG2 cells were incubated with atorvastatin, as was *CYP3A5*, though to a lesser extent [15].

Analysis of mRNA expression in peripheral blood mononuclear cells (PBMCs) has been used to evaluate the effects of lipid-lowering drugs on peripheral tissues *in vivo* [16,17]. Considering their involvement in pathophysiological mechanisms of cardiovascular-related diseases, mainly through the inflammatory pathways, expression in mononuclear cells could be used to evaluate modification of expression observed with this system [18].

In this study, we investigated the effects of atorvastatin on PBMCs *CYP3A4* and *CYP3A5* mRNA expression and CYP3A activity *in vivo*, measured by 6-beta-hydroxy-cortisol/cortisol ratio, and evaluated the interaction with common genetic variants of *CYP3A4* and *CYP3A5* in hypercholesterolemic individuals.

2. Subjects and methods

2.1. Subjects and study protocol

The characteristics of this study design and protocol have been previously reported [16,19,20]. Briefly, 458 individuals were selected to participate in the study at two clinical research centers in Sao Paulo, Brazil. Participants were screened and determined to be hypercholesterolemic (HC) with LDL cholesterol levels higher than 160 mg/dL ($n = 210$) or normolipidemic (NL; $n = 248$). Participants with hypertriglyceridemia (triglycerides $\geq$ 400 mg/dL), hypothyroidism, diabetes mellitus, liver or kidney diseases, and other forms of secondary dyslipidemia were excluded from this study. Overall 121 HC and 92 NL individuals consented and completed the study protocol. HC patients went through a low fat diet (according to American Heart Association Step One Diet) for four weeks [21]. Individuals who still had LDL cholesterol greater than 160 mg/dL were treated with atorvastatin 10 mg orally once daily for four weeks. HC patients were evaluated for serum concentrations of lipids to assess response to atorvastatin treatment, and alanine aminotransferase (ALT) and creatine kinase (CK) to monitor liver and muscle toxicity side effects. Patients continued taking other medications throughout the study, including diuretics, angiotensin converting enzyme (ACE) inhibitors, beta-adrenergic blockers, and calcium channel blockers.

Information on age, body mass index (BMI), blood pressure, menopause status, hypertension, obesity, family history of cardiovascular disease (CVD), tobacco smoking, alcohol consumption, exercise and current medications were recorded.

The study protocol was approved by the Ethics Committees of the Dante Pazzanese Institute of Cardiology, University Hospital and School of Pharmaceutical Sciences at the University of Sao Paulo, Sao Paulo, Brazil.

2.2. Samples

Blood samples were collected after a 12 hour fast. Patients undergoing atorvastatin treatment had blood drawn prior to and after the 4 week treatment. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured using routine enzymatic methods. CK and ALT were measured by kinetic methods. LDL and very low-density lipoprotein (VLDL) cholesterol were calculated [22]. Plasma apolipoprotein AI (apoAI) and apolipoprotein B (apoB) were determined by nephelometry.

Morning urine samples were collected. Urinary creatinine was measured by the Jaffe method. An aliquot was frozen at −80 °C for 6-beta-hydroxy-cortisol (6BOHC) and cortisol detection by high performance liquid chromatography (HPLC).

2.3. CYP3A4 and CYP3A5 genotyping

Genomic DNA was isolated from 1 mL EDTA-anticoagulated whole blood samples using a salting-out method [23]. *CYP3A4**1B (rs2740574) was detected by polymerase chain reaction (PCR) followed by restriction fragment analysis (RFLP) as previously published [24]. *CYP3A5**3C (rs776746) and *CYP3A4**22 (rs35599367) were detected using TaqMan Genotyping Assays [Life Technologies (Foster City, CA), catalog numbers C__26201809_30 and C_593013445_10]. Each PCR reaction contained 4 μL of Universal Master Mix (2×) (Life Technologies), 0.4 μL of the specific Genotyping Assay (20×), and 20 ng of DNA. The real-time PCR assays were carried out in 96-well plates using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal cycling protocol consisted of an initial cycle of 10 min at 95 °C followed by 40 cycles at 92 °C for 15 s, 60 °C for 1 min using standard 7500 conditions. *CYP3A5**1D (rs15524) was detected by DNA sequencing. Primer sequences and PCR thermal cycling conditions were previously published [20].

For quality control purposes, 5% of all samples were repeated and genotype accuracy was confirmed by DNA sequencing using the capillary electrophoresis system Mega BACE 1000 (Amersham Pharmacia Biotech, Upsala, Sweden) after PCR amplification using sets of primers designed with Primer Premier® v. 5.0 (Premier Biosoft International, USA).

2.4. RNA extraction and CYP3A4/CYP3A5 mRNA expression by real-time PCR

EDTA-anticoagulated blood was diluted in phosphate-buffered saline (1:1) and this suspension was layered in Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 30 min at 400 *g* at room temperature. PBMCs were collected from the interphase and immediately underwent RNA extraction using TRIzol® reagent (Invitrogen Corporation, CA, USA) according to the manufacturer's instructions.

Complementary DNA (cDNA) was produced from 1 μg of total RNA by SuperScriptTM II Reverse Transcriptase RNase H- with 10 μM random primers (Invitrogen Corporation, CA, USA) and *CYP3A4* and *CYP3A5* mRNA were measured by a TaqMan quantitative PCR assay.

The reference gene was chosen after analysis using the software geNorm [25]. Five reference genes were tested [hypoxanthine phosphoribosyl transferase I (*HRPT1*), hydroxymethyl-bilane synthase (*HMBS*), beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ubiquitin C (*UBC*)]. The most stable gene for PBMCs under experimental conditions was *GAPDH*, so it was used as a reference gene. The real-time PCR assays were carried out in 96-well plates using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). *CYP3A4* primer sequences were: F: TCAATAACAGTC TTTCCATTCCTCAT and R: TTCGAGGCGACTTTCTTTCATC, and the probe sequence was FAM™ TGTTTCCAAGAGAAGTTACMGBNFQ. For *CYP3A5*, primers were F: CTATCGTCAGGGTCTCTGGAAATT and R: ACGTTCCC CACATTTTTCCATA and the probe sequence was FAM™ACACAGAGT GCTATAAAAMGBNFQ. For *GAPDH*, primers were F: GGAAGGTGAAG GTCGGAGTCA, R: CTGGAAGATGGTGATGGGATTTC and the probe was VIC®TCAGCCTTGACGGTGC MGBNFQ. The thermal cycler protocol