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Characterization of simvastatin acid uptake by organic anion transporting polypeptide 3A1 (OATP3A1) and influence of drug-drug interaction

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ABSTRACT

Human organic anion transporting polypeptide 3A1 (OATP3A1) is predominately expressed in the heart. The ability of OATP3A1 to transport statins into cardiomyocytes is unknown, although other OATPs are known to mediate the uptake of statin drugs in liver. The pleiotropic effects and uptake of simvastatin acid were analyzed in primary human cardiomyocytes and HEK293 cells transfected with the OATP3A1 gene. Treatment with simvastatin acid reduced indoxyl sulfate-mediated reactive oxygen species and modulated OATP3A1 expression in cardiomyocytes and HEK293 cells transfected with the OATP3A1 gene. We observed a pH-dependent effect on OATP3A1 uptake, with more efficient simvastatin acid uptake at pH 5.5 in HEK293 cells transfected with the OATP3A1 gene. The Michaelis-Menten constant (K_m) for simvastatin acid uptake by OATP3A1 was 0.017 ± 0.002 μM and the V_{max} was 0.995 ± 0.027 fmol/min/10^5 cells. Uptake of simvastatin acid was significantly increased by known (benzylpenicillin and estrone-3-sulfate) and potential (indoxyl sulfate and cyclosporine) substrates of OATP3A1. In conclusion, the presence of OATP3A1 in cardiomyocytes suggests that this transporter may modulate the exposure of cardiac tissue to simvastatin acid due to its enrichment in cardiomyocytes. Increases in uptake of simvastatin acid by OATP3A1 when combined with OATP substrates suggest the potential for drug-drug interactions that could influence clinical outcomes.

1. Introduction

Cardiovascular disease is the primary cause of mortality in developed countries. Atherosclerosis, or plaque buildup inside the arteries, is a major underlying cause of cardiovascular disease. Evidence has shown a clear association between increased plasma cholesterol levels and atherosclerotic disease (Sytkowski et al., 1990; Gordon and Kannel, 1971). Hallmark studies including the Scandinavian Simvastatin Survival Study (4S), Heart Protection Study (HPS), Cholesterol and Recurrent events (CARE), and the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA) have evaluated the clinical efficacy of statins in managing cardiovascular risk and have shown reductions in cardiac mortality, coronary events, and low-density lipoprotein levels (Scandinavian Simvastatin Survival Study Group, 1994; Heart Protection Study Collaborative Group, 2002; Pfeffer et al., 1995; Sever et al., 2003). The kidney disease patient population has considerably higher rates of cardiovascular disease per age matched controls and the role of uremic toxins on this phenomenon has been hypothesized (Koukoulaki et al., 2012). Trials of lipid lowering in patients with kidney disease have shown variable successes [reviewed in Navaneethan et al. (2006)].

The primary mechanism of action for statins is to reversibly inhibit HMG-CoA reductase in the liver resulting in reduced hepatic cholesterol synthesis. Studies have shown that statins also possess numerous pleiotropic properties, independent of cholesterol lowering, which include nitric oxide-mediated improvement of endothelial dysfunction (Akalin Ciftci et al., 2015; Koh, 2000), antioxidant effects (Akalin Ciftci et al., 2015; Jaikumkao et al., 2016; Costa et al., 2016), anti-inflammatory properties (Akalin Ciftci et al., 2015; Garg et al., 2015; Mantuano et al., 2007), inhibition of cell proliferation (Lee et al., 2016; Yang et al., 2016; Lim et al., 2015), and stabilization of atherosclerotic plaques (Liao and Laufs, 2005; Kapur and Musunuru, 2008; Ueno et al., 2015). The observation of pleiotropic effects suggests that statins are a potential therapeutic option in several medical conditions for which they are not currently used. Simvastatin is a widely used anti-cholesterol drug in clinical practice, is generally well tolerated, and has a good safety profile. Studies have shown that simvastatin may be successful in improving endothelium-dependent vasodilation, increasing activity of vascular antioxidant systems, and reducing inflammatory markers (Mantuano et al., 2007; Landmesser et al., 2005; Carloni et al.,

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2006).

Simvastatin lactone metabolism is mediated by CYP3A4/5 in the intestinal wall and liver to form the active metabolite simvastatin acid, (pKa = 5.5) (Neuvonen et al., 2008). Uptake and efflux transporters localized to the basolateral and apical membrane of hepatocytes move simvastatin (active and inactive metabolites) from the portal circulation to the hepatocyte to bile, respectively (Neuvonen et al., 2008). Although the primary pharmacological site of action for cholesterol lowering is the liver, knowledge of the extrahepatic disposition of statins is relevant to our understanding of the off-target pleiotropic effects of these compounds. Since indoxyl sulfate is a uremic toxin implicated in the progression of renal and vascular diseases, we were interested in evaluating how this toxin influences OATP transport and cellular viability. This is also important to evaluate since transporter proteins are responsible for the removal of endogenous solutes from the brain, kidney, and liver, and increased levels of indoxyl sulfate can impair the ability of these transporters to remove other solutes that may contribute to systemic toxicity (Mutsaers et al., 2011; Tsujimoto et al., 2012; Ohtsuki et al., 2002).

Transporter proteins mediate the cellular uptake and efflux of a broad variety of endogenous compounds, drugs, and their metabolites and determine intracellular exposures (Mizuno et al., 2003). Organic anion transporting polypeptides (OATPs) are membrane influx transporters involved in the hepatic uptake of statins [reviewed in Roth et al. (2012)]. The role of transporters in the exposure of cardiac tissue to statins and their subsequent pleiotropic effects is unknown. Grube et al. identified and characterized OATP2B1 as a high-affinity transporter of atorvastatin in human heart tissue (Grube et al., 2006). OATP3A1/*SLCO3A1* mRNA levels have been detected in human heart, as well as testes and brain (Adachi et al., 2003; Huber et al., 2007). In rat hearts, significant OATP3A1 immunostaining was detected on cardiac muscle cells, endothelial cells of the left ventricular endocardium, and endothelial cells of the coronary artery (Adachi et al., 2003).

In this study, we investigated the ability of simvastatin acid to inhibit the production of reactive oxygen species (ROS) by the uremic toxin, indoxyl-3-sulfate. We also examined the expression of OATP3A1 in human cardiomyocytes and response to simvastatin acid exposure. Lastly, we characterized the *in vitro* uptake of simvastatin acid in transfected HEK293 cells expressing the human OATP3A1 transporter as a potential substrate of OATP3A1.

2. Materials and methods

2.1. Materials

[^{3}H]Simvastatin acid (10 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Simvastatin lactone and indoxyl-3-sulfate were purchased from Sigma Aldrich (St. Louis, MO). Inactive simvastatin lactone was activated as previously described (Sadeghi et al., 2000). Radioimmunoprecipitation assay (RIPA) Lysis Buffer, OATP3A1 antibody, and donkey anti-goat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin was purchased from Cell Signaling Technology (Beverly, MA). Pierce™ BCA Protein Assay Kit, SuperSignal® West Pico Luminol enhancer solution and SuperSignal® West Pico Stable peroxide solution were purchased from ThermoFisher Scientific (Rockford, IL). Mini-PROTEAN® TGX™ Precast gels 4–20% and 0.2 μm nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Unless specified, all other chemicals and reagents were obtained from Sigma Aldrich (St. Louis, MO).

2.2. Cell culture

Human cardiomyocytes (hCMs) from normal human ventricle tissue of the adult heart were obtained from PromoCell (Heidelberg, Germany) and maintained in Myocyte Growth Medium supplemented with fetal calf serum (0.05 mg/ml), epidermal growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), and insulin (5 μg/ml) (PromoCell). The hCMs express markers of early-stage differentiation, including GATA-4, sarcomeric α-actin, and display properties similar to progenitor cells with the capacity for proliferation. When grown to confluency, late differentiation markers are expressed, including slow muscle myosin and α-actinin. HEK293-NEO (empty vector) and HEK293-OATP3A1 transfected cells were kindly provided by Dr. Jörg König (Friedrich-Alexander-Universität Erlangen-Nurnberg,Erlangen, Germany) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, Rockford, IL) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 800 μg/ml Geneticin Selective Antibiotic (G418). All cells were cultured in a humidified 37 °C incubator with 5% CO_2. Unless specified, cells were treated with simvastatin acid for 24 h prior to experiments.

2.3. Assessment of dichlorofluorescein diacetate (DCFDA) fluorescence

The DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK) was used to assess intracellular ROS formation in cultures of cardiomyocytes as per manufacturer specifications. Briefly, hCMs were plated (25,000 cells) on dark, clear bottom 96-well microplates and incubated overnight. The hCM cells were treated with indoxyl sulfate (100–1000 μM), simvastatin (0.1–20 μM) + indoxyl sulfate (100–1000 μM), or diluent (vehicle control) for 24 h. Positive control cells were treated with TBHP (500 μM) for 4 h prior to treatment completion. Forty-five minutes prior to treatment completion, cells were stained with DCFDA solution (20 μM) and incubated at 37 °C in the dark. After incubation, fluorescence was measured using a BioTek Synergy™ 4 Hybrid Microplate Reader (Winooski, VT) using Gen5 1.10 software (BioTek) at excitation/emission 485/535 nm.

2.4. Western blot analysis

The hCMs and HEK293 cells transfected with OATP3A1 were lysed with RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentration was measured using Pierce™ BCA Protein Assay Kit. Protein (30 or 50 μg protein/well) was separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes by rapid transfer (Bio-Rad). After blocking with 10% nonfat dry milk in Tris-buffered Saline (TBS) with 0.5% of Tween 20 (TBST), membranes were incubated with primary antibody against OATP3A1 (sc-66566) in a 1:100 dilution of 1% nonfat dry milk in TBST overnight at 4 °C followed by donkey anti-goat IgG-horseradish peroxidase (HRP) (sc-2020) in a 1:2000 dilution for 1 h at room temperature. The SuperSignal Chemiluminescent Substrate (Thermo Scientific) was applied to the membrane prior to detection of luminescence using Image Lab v.5.2.1 (Bio-Rad). β-Actin (Cell Signaling) was used as a loading control.

2.5. OATP3A1 functional assays

The fluorescent substrate sodium fluorescein was used for characterization of OATP3A1 function in hCMs, HEK293-NEO (empty vector), and HEK293-OATP3A1-transfected cells. Cells (500,000 cells/dish) were grown on 100 mm dishes. Sodium fluorescein is a general substrate of OATP3A1 (Patik et al., 2015). Cells were pre-incubated with Dulbecco's Phosphate Buffered Saline (DPBS) pH 7.4 and pH 5.5 for 10 min at 37 °C in a 5% CO_2 atmosphere as several studies have reported increased OATP transport activity at acidic pH (Kobayashi et al., 2003; Nozawa et al., 2004; Varma et al., 2011). Cells were then treated with sodium fluorescein (2 μM) for 30 min (*uptake period*) (Wen et al., 2014). Only hCMs were incubated in the presence or absence of cyclosporine (suspected transport inhibitor) (10 μM) or simvastatin acid (10 μM) at 37 °C in a 5% CO_2 atmosphere for 30 min. After washing with DPBS pH 7.4 or 5.5, all cells were collected and lysed with 0.1 N NaOH and the intensity of fluorescence was quantified on a BioTek