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Methods

Statins alter the hepatobiliary transport of unconjugated and conjugated bilirubin in sandwich-cultured rat hepatocytes

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ABSTRACT

Several studies have reported that statins occasionally cause impairment of liver functions characterized by elevated serum bilirubin levels, which might be due to altered function of the multidrug resistance-associated proteins (Mrp2/3). We aimed to study the modulation of the hepatobiliary transport of bilirubin by four statin derivatives, atorvastatin, fluvastatin, pravastatin, and rosuvastatin in sandwich-cultured rat hepatocytes. All statins except pravastatin significantly inhibited the uptake of bilirubin. The biliary efflux of bilirubin conjugates was increased by pravastatin and rosuvastatin concentration dependently. Rosuvastatin stimulated not only the Mrp2 mediated biliary, but the Mrp3 mediated sinusoidal elimination, resulting in decreased intracellular bilirubin accumulation. The significantly induced Mrp2/3 protein levels (ranging from 1.5 to 1.8-fold) accounted for the elevated efflux. Cell polarization, the formation of biliary network was also significantly increased by fluvastatin, pravastatin and rosuvastatin (151%, 216% and 275% of the control, respectively). The simultaneous inhibition of the uptake and the stimulation of the sinusoidal and canalicular elimination may explain, at least in part, the clinical observation of elevated serum bilirubin levels. In conclusion, our results suggest that in spite of the elevated serum bilirubin levels, the altered Mrp2 and Mrp3 functions by statins is probably not associated with hepatotoxic effects.

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1. Introduction

Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as statins, are one of the most broadly used cholesterol lowering compounds (Hebert et al., 1997; Lewis et al., 1998; Maron et al., 2000). Although statins have a good safety profile, orally administered statins have been reported to induce prolonged (Bruggisser et al., 2010; Clarke and Mills, 2006; Famularo et al., 2007; Geoghegan et al., 2004; Merli et al., 2010) or even fatal cholestasis (Clarke and Mills, 2006; Perger et al., 2003; Sreenarasimhaiah et al., 2002) in some individuals associated with elevated serum aminotransferase and bilirubin levels. The mechanisms by which statins induce hepatic adverse changes are not known. Cholestasis is referred by jaundice with a concurrent elevation in alkaline phosphatase, γ-glutamyl transpeptidase (GGT), and conjugated bilirubin with little or no impairment in serum transaminase values (Holt and Ju, 2006; Mohi-ud-din and Lewis, 2004). However, any persistent abnormality in liver transaminases levels should be treated with caution. The elevation of serum aminotransferase in human does not appear to correlate with hepatotoxicity (Bader, 2010) and mild-to-moderate elevations in serum levels of liver transaminases constitute a regular response in patients undergoing statin therapy (Armitage, 2007; Chalasani et al., 2004; Jacobson, 2006; Parra and Reddy, 2003; Talbert, 2006; Vuppalanchi et al., 2005). Moreover, Bjornsson and Olsson (2005) confirmed that bilirubin was the only reliable indicator of drug induced cholestatic liver injury. It is not possible to establish causality between statin treatment and systemic bilirubin levels based on available data (Mol et al., 1988;

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, bovine serum albumin; Bsep, bile salt export pump; CDF, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein; CDFDA, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HBSS, Hanks' Balanced Salt Solution; GGT, γ-glutamyl transpeptidase; HMG-CoA, hydroxy-3-methylglutaryl coenzyme A reductase; HMOX1, heme oxygenase-1; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MRP, multidrug resistance-associated protein; OATP, organic anion-transporting polypeptide; SCRH, sandwich-cultured rat hepatocytes; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UGT1A1, UDP-glucuronosyltransferase 1A.

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Muchova et al., 2007; Ong et al., 2003). Presently, there is no information concerning the impact of statin treatment on bilirubin hepatic disposition.

In the blood circulation, bilirubin is mostly unconjugated and is tightly bound to albumin (Brodersen, 1980; Tenhunen et al., 1968). Bilirubin is taken up rapidly by hepatocytes partly by facilitated diffusion (Iga et al., 1979; Wolkoff et al., 1979 and Zucker et al., 1999), and partly mediated by organic anion transporter proteins (in humans, OATP1B1 and OATP1B3; in rodents, Oatp1a4) (Briz et al., 2006; Cui et al., 2001; Konig et al., 2000; Reichel et al., 1999; Roy Chowdhury et al., 2001). Bilirubin is extensively metabolized to mono- and diglucuronide conjugates by UDP-glucuronosyltransferase 1a1 (UGT1A1) (Bosma et al., 1994; Burchell, 1981; Halac and Sicignano, 1969). Under conditions of normal liver function, bilirubin conjugates are eliminated across the canalicular membrane into bile canaliculus via multidrug resistance-associated protein 2 (human/rodent; MRP2/Mrp2) (Jedlitschky et al., 1997; Kamisako et al., 1999; Keppler and Konig, 2000), the major determinant of bile salt independent bile flow (Paulusma et al., 1999; Trauner et al., 1998). When canalicular secretion is impaired, sinusoidally expressing MRP3/Mrp3 facilitates the release of toxic compounds like bilirubin and bilirubin conjugates from hepatocytes into sinusoidal space for their excretion via an alternative way through the kidneys to limit cell injury (Donner and Keppler, 2001; Konig et al., 1999; Ogawa et al., 2000; Teng and Piquette-Miller, 2007; Vos et al., 1998). Another cytoprotective mechanism in case of impaired biliary efflux is the downregulation of the uptake transporters (Le Vee et al., 2009). These processes may explain the accumulation of bilirubin and conjugated bilirubin in the blood in drug-induced liver injury. Therefore, statin-mediated hepatotoxic effects may result from the inhibition of hepatic uptake and/or the biliary efflux of bilirubin and conjugated bilirubin.

Sandwich-cultured rat hepatocytes (SCRH) were used as an *in vitro* model to assess the hepatotoxic potential of statins. Presently, only hepatocytes cultured in sandwich configuration are suitable for the study of a two directional transport of endogenous substances (including bilirubin and its conjugates) and drugs. SCRH is closer to *in vivo* hepatocyte structure compared to hepatocytes in conventional culture with re-established hepatic polarity, morphologically and functionally formed bile canaliculi and maintained key hepatic functions (Berthiaume et al., 1996; Dunn et al., 1991; LeCluyse et al., 2000).

In this study, we investigated the effects of multiple administrations of atorvastatin, fluvastatin, pravastatin, and rosuvastatin on the functions of Mrp2 and Mrp3, involved in bilirubin disposition, in addition to the assessment of Mrp2, Mrp3 and Oatp1a4 expression by Western blotting. Furthermore, we examined the modulation of Mrp2 function by quantifying the biliary excretion of 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF), a commonly used Mrp2 probe substrate, in statin-treated hepatocytes.

2. Methods

2.1. Materials

Atorvastatin and rosuvastatin were kindly provided by Gedeon Richter Plc. (Budapest, Hungary). Fluvastatin and pravastatin were a generous gift from Solvo Biotechnology (Szeged, Hungary). Matrigel was from BD Biosciences (Bedford, MA). All chemicals, including bilirubin, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), type IV collagenase, all cell culture media and reagents were purchased from Sigma–Aldrich (Budapest, Hungary). All other chemicals and reagents were of analytical grade and were readily available from commercial sources unless stated otherwise in the text. Stock solutions of test compounds were prepared in DMSO. Bilirubin (60 mM) was dissolved in DMSO/1.0 M NaOH (88:12) as a stock solution and diluted to 3 mM in BSA (20 mg/ml). The final bilirubin concentration was 10 μM in Williams' Medium E. Six-well plates were obtained from Greiner Bio-One (Mosonmagyarovar, Hungary). Sterile collagen from rat tail was prepared in-house according to established procedures.

2.2. Isolation and treatment of sandwich-cultured rat hepatocytes

Hepatocytes from adult male Wistar rats (Charles River, Budapest) weighing 200–250 g were isolated by a previously described three-step collagenase perfusion method (Lengyel et al., 2005; Seglen, 1976). The protocol was approved by the Institutional Animal Care and Use Committee (Permit Number: 22.1/2728/3/2011). All surgeries were performed under diethyl ether anaesthesia, and all efforts were made to minimize suffering. Cell viability (>90%) was routinely checked by the trypan blue exclusion test. After preparation, freshly isolated cells were suspended in Williams' Medium E and plated at a cell density of 2×10^6 cells/well in six-well Greiner culture plates previously coated with rat tail collagen. The cells were initially maintained in Williams' Medium E supplemented with 5% of fetal calf serum (FCS), 0.1 μM insulin, 0.05 μM glucagon, 0.1 mg/ml gentamicin, 0.03 μM Na_2SeO_3, and 0.1 μM dexamethasone and allowed to attach for 1 h at 37 °C in a humidified incubator with 95% air/5% CO_2. After the cell attachment, the culture dishes were gently swirled, and the medium was replaced. Calf serum was present for the first 24 h, then omitted. In order to achieve sandwich configuration, at 24 h after plating, the medium was aspirated and the cells were overlaid with Matrigel basement membrane matrix at a concentration of 0.25 mg/ml in 1.5 ml of ice-cold serum-free Williams' Medium E. The culture medium was replaced every 24 h and the studies were conducted on day 4 of culture.

Following the cell attachment, the hepatocytes were treated with various concentrations (0.1, 1 and 10 μM) of atorvastatin, fluvastatin, pravastatin and rosuvastatin or the vehicle (0.1% DMSO) as control daily for 3 days. Each statin concentration was tested in three independent hepatocyte preparations using three wells/treatment groups with each preparation.

2.3. Hepatobiliary disposition of bilirubin and conjugated bilirubin in SCRH

Following the statin treatments of SCRH, on day 4, the hepatobiliary transport of bilirubin and its conjugates was measured as described earlier (Lengyel et al., 2005). Briefly, the culture medium was removed and the cells were washed with HBSS buffer containing calcium (standard buffer) at 37 °C. The experiment was started by the addition of 1 ml pre-warmed standard buffer containing 10 μM of bilirubin to all wells for 5 min at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. After the hepatocytes were loaded with bilirubin, the loading medium was removed, and the cells were washed vigorously three times with 1 ml of ice-cold standard buffer or HBSS without Ca^{2+}/Mg^{2+}, containing1 mM EGTA (calcium-free buffer) to stop bilirubin uptake. Thereafter, the hepatocytes were incubated with standard or calcium-free efflux medium (HBSS, representing the sinusoidal space) for 10 min. The efflux process was terminated by the removal of the efflux medium, and the wells were rinsed with ice-cold standard buffer. Then, the cells were lysed with 1 ml of an acetonitrile/water solution [30% (v/v)] by shaking for 20 min at 0 °C and sonicated for an additional 5 min. The amounts of bilirubin and bilirubin conjugates in the efflux medium and in the cell lysates were analyzed by a high-performance liquid chromatography (HPLC) system. Data were normalized to the protein content, which was determined by the method of Lowry et al. (1951). Incubation in standard buffer maintains the integrity of

the tight junctions sealing bile canalicular networks, whereas incubation in calcium-free buffer disrupts the tight junctions. The transport of bilirubin and bilirubin conjugates into the bile canaliculi was determined by subtracting the amount of bilirubin and its conjugates in standard buffer from that in calcium-free buffer and expressed as nmol/mg protein. Each statin concentration was tested in three independent hepatocyte preparations using three wells/treatment groups with each preparation.

2.4. HPLC measurement of bilirubin and conjugated bilirubin

The HPLC method for analyzing bilirubin and conjugated bilirubin was performed as described previously (Lengyel et al., 2005) with modifications. In brief, samples were centrifuged at 13,000 rpm for 10 min, and 200 μl of supernatant was analyzed on a Merck/Hitachi HPLC system. Bilirubin and bilirubin conjugates were resolved on a Chromolith Performance RP 18e (4.6 × 100 mm, Merck Darmstadt, Germany) reversed-phase chromatographic column. Elution was achieved at 3 ml/min flow rate using a binary gradient of solvent A (75% 0.01 M sodium phosphate buffer, pH 3.2 and 25% acetonitrile, containing 200 μl/l triethylamine) and solvent B (acetonitrile) and a total run time of 10 min. After 1 min of isocratic elution with solvent A, the gradient started reaching 10% of eluent B in 5.2 min. Following the separation of the conjugates, the column was rinsed of any remaining hydrophobic components by using a gradient reaching 100% eluent B at 7.5 min, and then the column equilibration time was 2.5 min. The UV absorbance was monitored at a wavelength of 450 nm. The amount of bilirubin and bilirubin conjugates were quantified using calibration curves prepared with bilirubin as standard. The transport rate was estimated by the slope of the regression of nanomoles of bilirubin or bilirubin conjugates excreted per milligram of hepatocyte protein.

2.5. Analysis of bile canaliculi formation and function

The bile canaliculi formation and function was verified by using 5 (and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDF-DA, Invitrogen), a specific Mrp2 substrate. The fluorescent CDF, a metabolite of CDF-DA is actively excreted into the bile canaliculi via Mrp2 (Zamek-Gliszczynski et al., 2003). Following the statin treatments of SCRH for 3 days the cells were incubated with 2 μM CDF-DA in standard buffer for 20 min at 37 °C. The morphology of hepatocytes and the accumulation of CDF in the bile canaliculi were observed with an upright epifluorescent microscope (Olympus BX61WI) equipped with a FluoView300 confocal laser-scanning system (Olympus, Tokyo, Japan) using 60× water immersion objective (numeric aperture, 0.9). The fluorescence measured with a green fluorescence of CDF excited at 488 nm was collected through a 510–530 nm bandpass filter. The interaction of statins with Mrp2-mediated CDF transport was evaluated by quantifying the accumulated fluorescence in the bile canaliculi using ImageJ software (http://rsb.info.nih.gov/ij/). The formation of bile canaliculi was determined as area % of canaliculi compared to the total area.

2.6. Western blot studies of Oatp1a4, Mrp2 and Mrp3

The amount of Oatp1a4, Mrp2 and Mrp3 protein was determined semi-quantitatively by Western blotting of total cell lysates. After 3 days of statin treatment (10 μM), the cells were washed tree times with ice-cold standard buffer and harvested in 200 μl of lysis buffer consisting of 0.1 M Tris–HCl, pH 8.0, and protease inhibitor cocktail. Anti-Oatp1a4 (M-50) and anti-Mrp3 (H-16) antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Mrp2 (M2III-5) antibody was purchased from Alexis Biochemicals (Lausanne, Switzerland). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma–Aldrich (Budapest, Hungary). The samples were normalized for protein content, and 60 μg of each sample were separated using 7.5% SDS–polyacrylamide gel and transferred to polyvinylidenedifluoride membrane (Immobilon-P; Millipore, Bedford, MA) at 350 mA in a transfer buffer composed of 25 mM Tris, 192 mM glycine, and 15% (v/v) methanol, pH 8.3. The membranes were treated with blocking buffer (5% nonfat dry milk powder and 0.5% BSA in phosphate-buffered saline with 0.05% Tween 20) for 2 h at room temperature. The membranes were then incubated with the primary antibody, a rabbit anti-Oatp1a4 monoclonal antibody, a mouse anti-Mrp2 monoclonal antibody or Mrp3 monoclonal antibody, diluted 1:500, 1:2000 or 1:1000 in blocking buffer for 2 h at room temperature. After extensive washes with phosphate-buffered saline/0.05% Tween 20 for 3 × 10 min at room temperature, the membranes were incubated with the secondary antibody, anti-rabbit or anti-mouse IgG-HRP, a horseradish peroxidase-conjugated species-specific whole antibody diluted 1:5000 in phosphate-buffered saline/0.05% Tween 20 for 1 h at room temperature. Subsequently, the membranes were washed as described above, and the immunoreactive bands were visualized with ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric scanning of protein bands was performed using ImageJ software.

2.7. Statistical analysis

Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Effects of statin treatment on the hepatobiliary transport of bilirubin and conjugated bilirubin

Following the uptake of bilirubin by hepatocytes, its conjugated metabolites are transported into the canalicular space and into the medium by Mrp2 and Mrp3, respectively. The modulation of these transport processes by multiple administrations of statins at different concentrations was evaluated in SCRH. Lack of toxicity of the statins in the experiments was proven by MTT test (data not provided). Fig. 1 summarizes the uptake and the total, two directional effluxes of bilirubin and conjugated bilirubin following a 3-day treatment by 10 μM statin derivatives. The uptake of bilirubin is presented as nmol/mg/5 min in Fig. 1A. Bilirubin uptake was 2.46 ± 0.20 nmol/mg/5 min in the control, which significantly decreased after treatment with atorvastatin, fluvastatin, and rosuvastatin (1.84 ± 0.34, 1.99 ± 0.38, and 1.48 ± 0.31 nmol/mg/5 min, respectively). Pravastatin did not alter the bilirubin uptake significantly (2.22 ± 0.39 nmol/mg/5 min). Statin treatments with lower concentrations (0.1 and 1 μM) did not cause significant difference in bilirubin uptake compared to control. Similarly there was no significant effect at the lower concentrations concerning hepatobiliary elimination of unconjugated and conjugated bilirubin obtained for atorvastatin, fluvastatin and pravastatin (data not shown). At 10 μM all statins but fluvastatin significantly increased the total elimination of bilirubin metabolites compared to the control (Fig. 1B). Though pravastatin did not alter the uptake, this drug increased the elimination of conjugated bilirubin by nearly 50%. Rosuvastatin treatment had the most pronounced effect on both the hepatic uptake (60% of the control) and the elimination (190% of the control) of bilirubin and its conjugates.

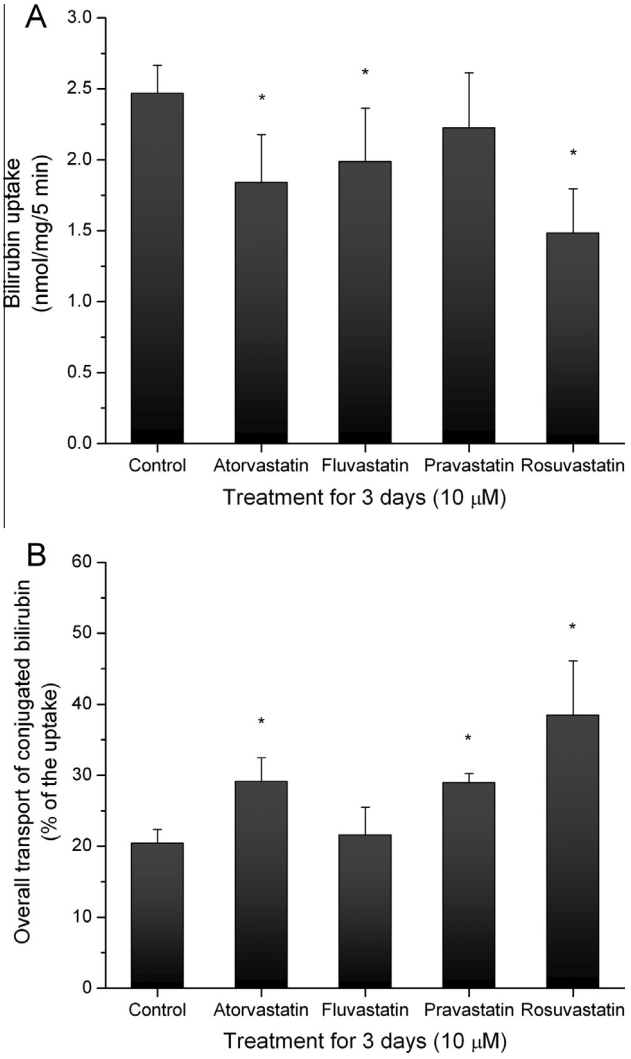

Fig. 1. The effects of statin treatment (10 μM) on the hepatic uptake of bilirubin (A) and on the overall transport of conjugated bilirubin (B). Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$.

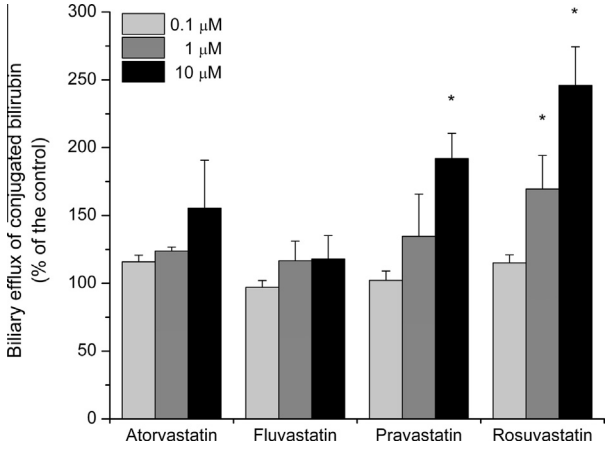

Fig. 2. The effects of statin concentration on the biliary efflux of conjugated bilirubin. The results are presented as percentage of the control. Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$.

The effect of statin concentration on the canalicular efflux of conjugated bilirubin is presented in Fig. 2. At 0.1 μM none of the statins influenced the Mrp2-mediated transport of conjugated bilirubin. Atorvastatin and fluvastatin had little and not significant effect at the higher concentrations tested. In contrast, a significant and concentration-dependent increase of the biliary efflux was observed in the pravastatin- and rosuvastatin-treated hepatocytes compared to the non-treated cells. The canalicular efflux of the bilirubin metabolites was elevated to 135% and 190% by 1 and 10 μM pravastatin and to 170% and 245 % by 1 and 10 μM rosuvastatin (compared to untreated cells), respectively. All statins tested in this study had no influence on the hepatic conjugation of bilirubin (data not shown).

The canalicular, sinusoidal disposition and the intracellular accumulation of conjugated bilirubin in control and statin treated (10 μM) hepatocytes are shown in Fig. 3. Data are expressed as the % of the uptake. The rate of net biliary and sinusoidal efflux of conjugated bilirubin was 0.58 ± 0.27 nmol/mg/10 min and 0.31 ± 0.13 nmol/mg/10 min in control cells. Atorvastatin and fluvastatin did not alter the elimination profile of bilirubin conjugates. Under the same conditions, treatment with pravastatin elevated although not significantly the biliary and decreased the sinusoidal efflux, resulting in the shift of the direction of elimination towards the canalicular space. In the control SCRH the canalicular transport was the double of the sinusoidal transport, and this difference increased to four fold by 10 μM of pravastatin. Rosuvastatin treatment elevated the elimination of bilirubin metabolites towards both directions (170% and 189% of the control, respectively). As a result, the intracellular accumulation of the conjugated bilirubin decreased by 46% changing the elimination profile significantly (Fig. 3). As the profile of conjugated bilirubin elimination observed after rosuvastatin treatment highly differed from that produced by any other statins examined, we focused on the concentration dependence of these effects. As shown in Fig. 4, rosuvastatin exhibited a concentration dependent inhibitory effect on hepatic uptake and a stimulating effect on the biliary efflux, which resulted in a significantly reduced intracellular bilirubin level.

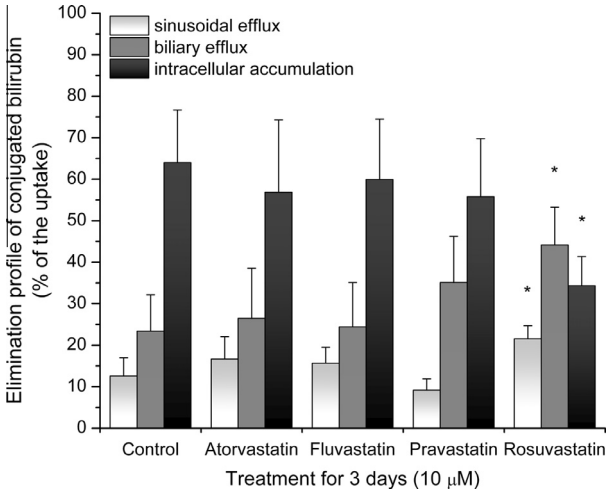

Fig. 3. The effects of statin treatment (10 μM) on the hepatic disposition of conjugated bilirubin. Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$.

3.2. Effects of statin treatment on the canalicular network formation

Since the Mrp2-mediated transport of bilirubin conjugates was modified by pravastatin and rosuvastatin treatment, we investigated the influence of statins on Mrp2 function applying another, Mrp2 specific substrate (CDF). Confocal laser scanning microscopy was used to illustrate the transport of CDF into the bile canaliculi. The fluorescence images showed that CDF was mainly localized in the canalicular networks after 20 min of incubation (Fig. 5A). The ImageJ software was applied in order to quantify the change of bile canaliculi formation (Fig. 5B). Atorvastatin-treated SCRH showed similar canalicular network density as the control cells. Fluvastatin slightly (151%) while pravastatin and rosuvastatin significantly (216% and 275% of the control) increased the length of the canaliculi. In addition, statin administration caused an apparent morphological change in the bile canaliculi network, resulting in large dilated canaliculi and an increase in canalicular branching.

3.3. Effects of statin treatment on the expression of Oatp1a4, Mrp2 and Mrp3

To determine whether statin-induced reduction of bilirubin uptake corresponded to altered protein expression of Oatp1a4, we measured the expression level of this transporter via Western blot hybridization. Fig. 6A shows a representative Western blot of statin-treated rat hepatocytes demonstrating that fluvastatin and rosuvastatin had a significant effect on Oatp1a4 expression level. Fig. 6B shows that the expression level of Oatp1a4 in fluvastatin-treated group was decreased by 35% with respect to the control, whereas in atorvastatin-treated group was only slightly decreased (12%). Data showed that rosuvastatin treatment resulted in a significant decrease in the amount of Oatp1a4 protein (70%) compared to the control.

The assumption that statin-induced elevation of the transport activity of Mrp2 and Mrp3 is associated with elevated protein levels was confirmed by Western blot hybridization. Representative Western blots of Mrp2 and Mrp3 expression following statin-treatment are shown in Fig. 7A and B. Densitometric analyses of the Western blots are shown in the lower panel (Fig. 7C). As expected, the treatment of SCRH with 10 μM pravastatin and rosuvastatin produced marked increases in the amount of Mrp2 and Mrp3 proteins (ranging from ~ 50% to 80%), whereas no discernible changes were observed by atorvastatin. There was a 37% increase in Mrp2 and a 32% increase in Mrp3 protein expression in fluvastatin-treated hepatocytes compared with the control by 72 h.

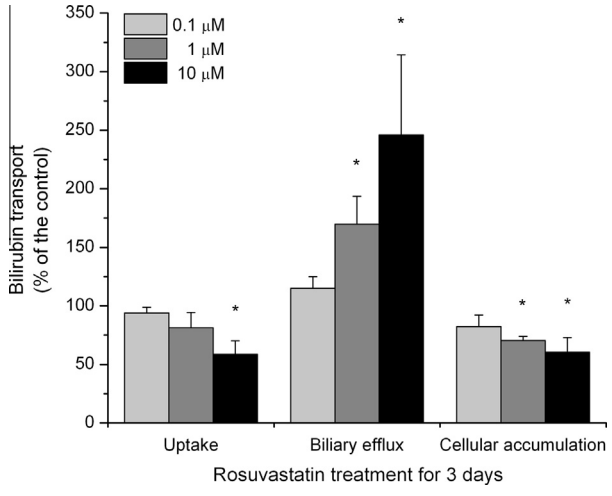

Fig. 4. Concentration dependent effects of rosuvastatin (up to 10 μM) on the hepatic disposition of bilirubin. Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$.

4. Discussion

Rather small knowledge is available concerning the expression and/or activity of MRP2 and MRP3 transporters in connection with statin-induced liver injury; however, the functional alteration of these transporters may explain the impaired hepatic trafficking of bilirubin, which is considered as a sensitive signal of hepatotoxicity. The down-regulation of Mrp2/MRP2 transporter has been characterized in both rodents and primary human hepatocytes (Kullak-Ublick et al., 2002; Trauner et al., 1997). Particularly, statins may limit the hepatic elimination of bilirubin by blocking different pathways associated with bilirubin disposition including both the uptake and the efflux processes. Our objective was to characterize the alteration of the hepatobiliary transport of bilirubin and its conjugates following multiple treatments with four statin derivatives, atorvastatin, fluvastatin, pravastatin, and rosuvastatin. In the present study, SCRH was applied as an *in vitro* model, in order to enable to examine the two directional transport processes involved, such as the uptake and the efflux on the sinusoidal and the efflux on the canalicular membrane surface. The methodology used allowed us to measure the uptake, the sinusoidal and the canalicular efflux and the intracellular accumulation of bilirubin and its conjugates, simultaneously. Preliminary viability studies confirmed that the statins applied in the drug interaction experiments were not cytotoxic for SCRH even at the highest concentration used. Statins were administered daily for 3 days in order to monitor their longer term effect on bilirubin elimination. Atorvastatin, fluvastatin, and rosuvastatin effectively inhibited the hepatic uptake of bilirubin, which is mostly taken up by Oatp1a4 (Fig. 1A) (Reichel et al., 1999; Roy Chowdhury et al., 2001). This observation directly correlates with our Western blot data on Oatp1a4 expression in statin-treated rat hepatocytes (Fig. 6). Furthermore, our results imply that these statins shared the same transporters as bilirubin and this might also cause the decrease of bilirubin uptake. This is in accord with previous reports indicating that Oatps play a crucial role in the uptake of statins in rats (Nezasa et al., 2003; Sakamoto et al., 2008; Sugatani et al., 2010; Watanabe et al., 2009). On the contrary, pravastatin did not alter the uptake rate of bilirubin. This can be attributed to the fact that pravastatin is a substrate for more Oatps, namely, Oatp1a1 and Oatp1b2 but not for Oatp1a4 (Bergwerk et al., 1996; Hsiang et al., 1999; Sasaki et al., 2004), which is involved in the uptake of bilirubin (Reichel et al., 1999; Roy Chowdhury et al., 2001). Moreover, administration of pravastatin (5 mg/kg) significantly increased the expression level of Oatp1a4 *in vivo* in rat liver (Kolouchova et al., 2011). However, both pravastatin and rosuvastatin are low permeable, hydrophilic drugs and are known to be excreted extensively into the bile as unchanged compounds in rats (Kitamura et al., 2005; Yamazaki et al., 1997); our results concerning the inhibitory effectiveness of the two drugs towards bilirubin uptake, also support that their uptake mechanisms might be different. This is in good agreement with previously published data, which showed that the uptake clearance of rosuvastatin was approximately 10-fold higher than that of pravastatin in rats (Nezasa et al., 2003). In addition, the uptake clearance of atorvastatin and fluvastatin determined in rat hepatocytes were also more than 10-fold higher compared to pravastatin (Watanabe et al., 2010).

Atorvastatin, pravastatin and rosuvastatin enhanced the two directional elimination of conjugated bilirubin (Fig. 1B). It is well published that xenobiotics often induce metabolic enzymes and

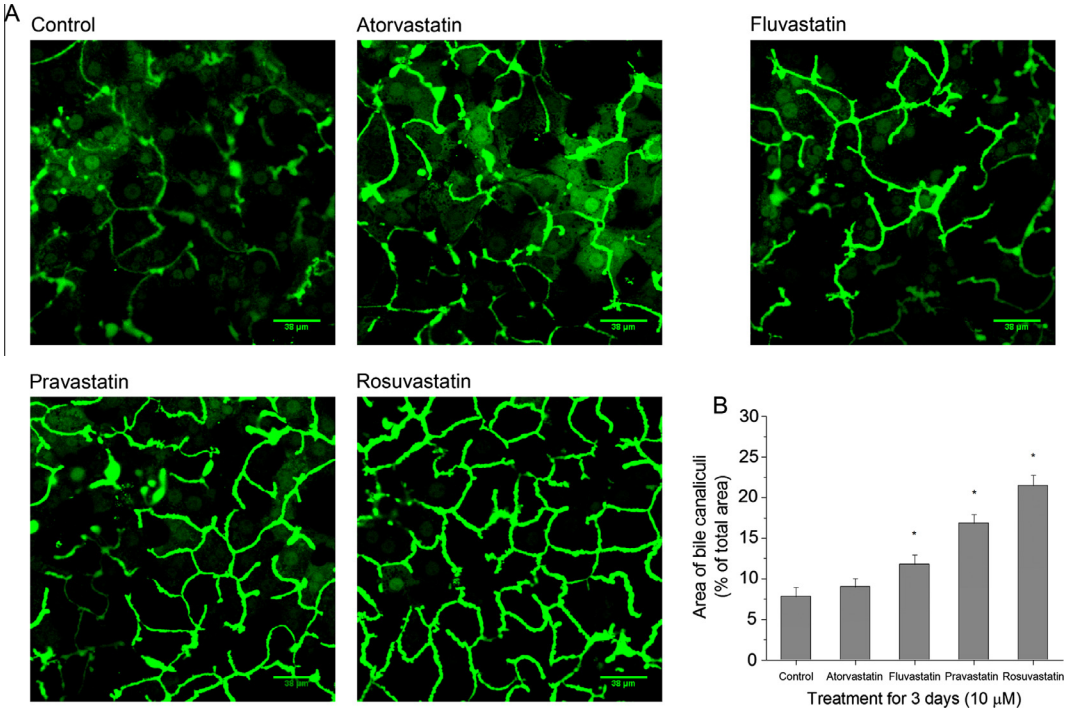

Fig. 5. The effects of statin treatment on the bile canaliculi network formation in SCRH. Hepatocytes were treated daily for 3 days with atorvastatin, fluvastatin, pravastatin and rosuvastatin (10 μM), or the vehicle (0.1% DMSO) as control. SCRH were incubated with 5 (and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) (2 μM) for 20 min at 37 °C under 5% CO_2 in air. In the cells, endogenous esterases cleave the cell-permeable dye. The resulting CDF is excreted into the bile canaliculi (bright green segments) via Mrp2. Fluorescence images (A) and corresponding fluorescent results (B). Data were determined in triplicate from three individual livers and are expressed as mean ± standard deviation (SD). Statistically significant differences in the uptake or the efflux of bilirubin and its conjugates due to statin treatments were calculated using Student's *t*-test. In all cases, the criterion for statistical significance was $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

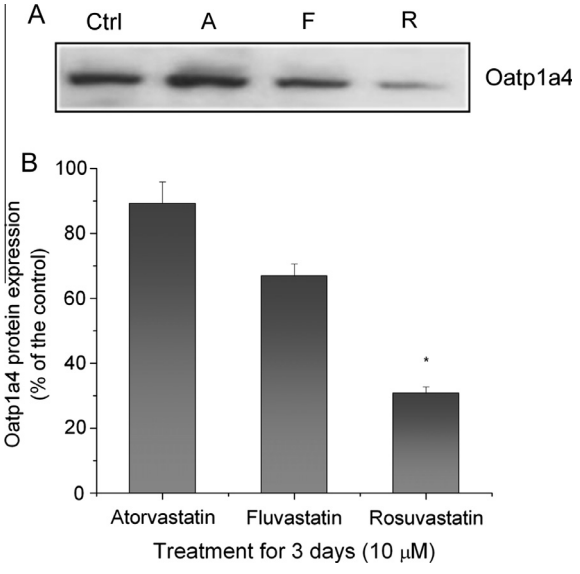

Fig. 6. The effects of statin treatment on the expression of Oatp1a4. Typical patterns of Western blot analysis of Oatp1a4 in statin-treated rat hepatocytes. Hepatocytes were treated daily for 3 days with atorvastatin, fluvastatin and rosuvastatin (10 μM), or the vehicle (0.1% DMSO) as control. Total proteins were extracted from the cells and western blot for Oatp1a4 (A) was performed. Mean densitometry measurements (± SD) from three individual livers (B). Results of the densitometric analysis are given as percentage of the controls. $^{*}P < 0.05$ statistically significantly different from the control. Ctrl, control; A, atorvastatin; F, fluvastatin; R, rosuvastatin; Oatp, organic anion-transporting polypeptide.

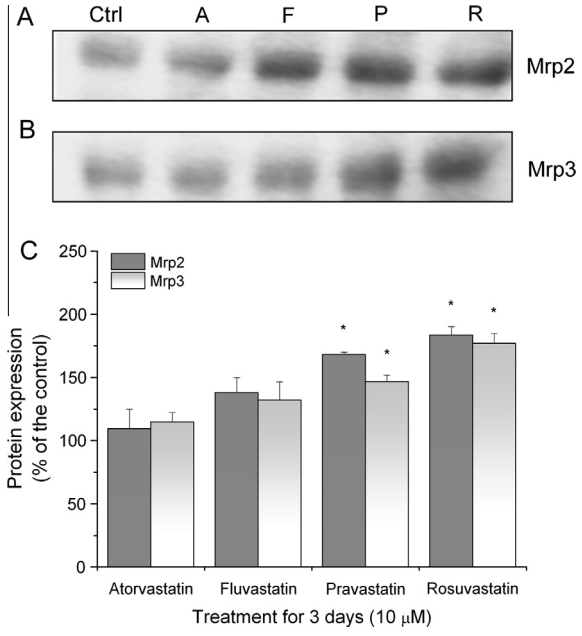

Fig. 7. The effects of statin treatment on the expression of Mrp2 and Mrp3. Typical patterns of Western blot analysis of Mrp2 and Mrp3 in statin-treated rat hepatocytes. Hepatocytes were treated daily for 3 days with atorvastatin, fluvastatin, pravastatin and rosuvastatin (10 μM), or the vehicle (0.1% DMSO) as control. Total proteins were extracted from the cells and western blots for Mrp2 (A) and Mrp3 (B) protein were performed. Mean densitometry measurements (±S.D) from three individual livers (C). Results of the densitometric analysis are given as percentage of the controls. $^{*}P < 0.05$ statistically significantly different from the control. Ctrl, control; A, atorvastatin; F, fluvastatin; P, pravastatin, R, rosuvastatin; Mrp, multidrug resistance-associated protein.

transporters involved in their elimination, which enables organisms to cope with the continuously changing chemical environment. Previous studies have demonstrated that all statins tested in this study are both substrates and inhibitors for Mrp2 in experiments using membrane and vesicle preparations, hepatocytes, and *in vivo* (Abe et al., 2008; Ellis et al., 2013; Yamazaki et al., 1997). At the same time, fluvastatin treatment did not affect the rate of bilirubin efflux, the interpretation of which might be that different transport processes are involved in the elimination of fluvastatin and bilirubin. This result is consistent with a previous study (Watanabe et al., 2010) revealing a minor contribution of any canalicular transporter to the elimination of fluvastatin in rats, since just 9% of the oral dose was recovered in the bile in male SD rats. In our study, the elevated expression of the Mrp2 protein in response to rosuvastatin and pravastatin treatments supports that the accelerated bilirubin efflux was due to the induction of this transporter (Fig. 7). In accord with our results, pravastatin treatment *in vivo* (5 mg/kg) significantly increased the expression level of Mrp2 in rats (Kolouchova et al., 2011).

Another important finding of the current study is the unique effect of statins on bile canalicular network formation (Fig. 5). The mechanisms by which statins regulate canalicular network formation are unknown yet; however, we think that it is connected with the increased Mrp2 expression. An analogue mechanism has been described by Fu et al. (2011), who showed that taurocholate, a substrate for another canalicular transporter (Bsep, bile salt export pump), stimulates hepatocyte polarization and accelerates the canalicular network formation in SCRH. Under the influence of statins, the number and size of bile canaliculi significantly increased and they formed a more complex, branching architecture. These bile canaliculi were functional as demonstrated by the Mrp2-mediated CDF transport. Summing up, statin treatment rather seems to induce choleresis than cholestasis, as it was demonstrated in Fig. 5. We believe that our observation is the first experimental evidence concerning the effect of statins on cell polarization and bile canaliculi formation.

Rosuvastatin was the only statin which remarkably stimulated both the sinusoidal and biliary efflux of conjugated bilirubin (Fig. 3). The results observed at the transport activity level could be detected also at the protein level (Fig. 7). The exact mechanisms involved in the increased expression of Mrp2 and Mrp3 are not known, and further investigations of this phenomenon are obviously required. Kobayashi et al. (2013) demonstrated that pitavastatin treatment induced the expression of MRP2/Mrp2 through an SREBP regulatory pathway in HepG2 cells and rat liver.

By inhibiting bilirubin uptake and stimulating unconjugated and conjugated bilirubin elimination, rosuvastatin may have a potential role in liver protection through maintaining intracellular bilirubin level low, as we have demonstrated (Fig. 3). The observed effect was concentration-dependent (Fig. 4). The inhibition of bilirubin uptake and the stimulation of the sinusoidal elimination of conjugated bilirubin by statins may explain, at least in part, the clinical observation of elevated serum bilirubin levels. It is generally assumed that the high hepatocellular concentration of bilirubin is the primary determinant of drug-induced hepatotoxicity. Based on our findings, elevated serum bilirubin levels may not necessarily be an appropriate indicator of hepatotoxicity associated with statin treatment. In other words, an increased serum bilirubin level not inevitably refers to the hepatotoxic potential of statins, since an elevated serum concentration does not correlate with higher intracellular bilirubin concentrations. In addition, Muchova et al. (2007) reported that the long-term use of statins induced heme oxygenase-1 (HMOX1) in mice and through the intervention of upregulated HMOX1 (conversion of heme into bilirubin) activity increased serum and tissue bilirubin concentrations *in vivo*. Additionally, serum bilirubin concentrations are influenced by many factors including, dietary, intake of numerous drugs, gender, race, and age (Vítek and Schwertner, 2007).

In summary, we have demonstrated for the first time that statins modulate bilirubin uptake and elimination. Our results indicate that rosuvastatin remarkably reduced the intracellular concentration of bilirubin and conjugated bilirubin by decreasing bilirubin uptake via Oatp1a4, increasing biliary excretion via Mrp2 and sinusoidal elimination via Mrp3. We showed that statins exerted their effect by simultaneously suppressing Oatp1a4 protein and inducing Mrp2, Mrp3 proteins. Rosuvastatin and pravastatin accelerated cell polarization and canalicular network formation in SCRH. Taken together, these results suggest that the alteration of Mrp2 and Mrp3 transport activity by statins is not associated with enhanced intracellular bilirubin concentration in rat hepatocytes. Consequently, the cholestatic cases reported probably were not due to intracellular hepatotoxicity caused by statin treatment.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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