ORIGINAL ARTICLE

The influence of Rosuvastatin on liver microsomal CYP2C6 in hereditary hypertriglyceridemic rat

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Abstract

OBJECTIVES: The aim of this study was to investigate whether rosuvastatin affects expression and activity of rat CYP2C6. This cytochrome P450 is considered to be a counterpart of human CYP2C9, which metabolizes many drugs, including diclofenac, ibuprofen or warfarin.

DESIGN: Male hereditary hypertriglyceridemic (HHTg) rats were fed standard laboratory diet (STD) or high cholesterol diet (HCD: STD + 1% of cholesterol w/w + 10% of lard fat w/w) for 21 days. A third group of rats were fed high a cholesterol diet with rosuvastatin added (0.03% w/w). Expression of CYP2C6 was measured in liver samples using real-time PCR (mRNA level) and Western blotting (protein level). Formation of diclofenac metabolites (typical enzyme activity of CYP2C6) was analyzed using HPLC with UV detection.

RESULTS: Administration of rosuvastatin to HHTg rats resulted in significantly increased mRNA expression and enzyme activity in HCD-fed animals; changes of CYP2C6 protein were non-significant. These results suggest that CYP2C6 expression and activity are positively affected by rosuvastatin in hereditary hypertriglyceridemic rats after intake of HCD.

CONCLUSION: The results presented open the possibility that in humans, rosuvastatin may affect the metabolism of many drugs by influencing expression and activity of CYP2C6 (counterpart of human CYP2C9). Further studies are needed to elucidate the effects of this statin on CYP2C9 in humans.

Abbreviation:

BCA	- bicinchoninic acid
cDNA	- complementary deoxyribonucleic acid
CAR	- constitutive androstane receptor
CYP	- cytochrome P450
HHTg	- hereditary hypertriglyceridemia
HMG-CoA	- 3-hydroxy-3-methylglutaryl-coenzyme A
HRP	- horseradish peroxidase
mRNA	- messenger ribonucleic acid
RT-PCR	- real-time polymerase chain reaction
SDS	- sodium dodecyl sulfate

INTRODUCTION

Hypercholesterolemia and/or hypertriglyceridemia are accepted risk factors for coronary heart disease. Rosuvastatin is one of the recently marketed fully synthetic HMG-CoA reductase inhibitors (statins) used in lipid lowering therapy (Kralova-Lesna *et al.* 2011). This widely used hypolipidemic drug exhibits high hepatoselectivity and undergoes only limited metabolism via cytochrome P450 (Neuvonen 2010; Kostapanos *et al.* 2010).

The risk of interactions generally rises with increasing dose and in concomitant administration with agents that may interact with the same metabolic or transport pathway (Rubba *et al.* 2009; Kostapanos *et al.* 2010). It should be noted that drug-drug interactions were identified as a leading cause of patient hospitalization and death (Jaquenoud *et al.* 2006).

In humans, rosuvastatin is metabolized (about 10% of the dose) via CYP2C9 and CYP2C19 (McTaggart *et al.* 2001). High importance of CYP2C9 in metabolism of commonly used drugs is well documented (warfarin, diclofenac, ibuprofen and others) (Mo *et al.* 2009). Current reports mention important interactions between rosuvastatin and other drugs metabolized by CYP2C9 in human (warfarin, sildenafil, glimepiride, telmisartan) (Gallelli *et al.* 2009; Galani & Vyas 2010; Pennisi *et al.* 2010).

Rat CYP2C6 is one of the main cytochromes P450 in rat liver microsomes (about 20% of the total CYP content). This form of cytochrome P450 can also be regarded as a counterpart of human CYP2C9 (Daniel *et al.* 2006). Human CYP2C9 and rat CYP2C6 are known to share substrates, e.g. they both catalyze the 4´-hydroxylation of diclofenac (Leemann *et al.* 1993), hydroxylation of tolbutamide (Jurica *et al.* 2009) and 7-hydroxylation of warfarin (Daniel *et al.* 2006).

Interestingly, we have recently found that in healthy (i.e. non-HHTg) rats on the high cholesterol diet rosuvastatin resulted in a decreased expression of CYP2C6 (at the level of mRNA without significant changes in the protein levels) and decreased activity of this cytochrome P450 (Zachařová *et al.* 2012). Our previous results have indicated that the expression of genes responsible for lipid homeostasis, including cytochromes P450, may be influenced by a cholesterol rich diet (Orolin *et al.* 2009). Taken together, the interplay of the lipid homeostasis, high dietary cholesterol and fat intake and effect of hypolipidemics (fibrates or statins) seem to alter the ability of organism to metabolize concomitantly taken drugs (Večeřa *et al.* 2011; Zachařová *et al.* 2012). High cholesterol diet (high content of cholesterol and saturated fatty acids), is used to simulate the unhealthy diet often seen in patients with metabolic syndrome (Charlton *et al.* 2011).

This study is aimed at investigating the effect of rosuvastatin (0.03 % w/w), administered in high cholesterol and high fat diet for 21 days, on the expression of liver CYP2C6 in hereditary hypertriglyceridemic male Wistar rats, which represent an established model of metabolic syndrome accompanied by hypertriglyceridemia, liver steatosis, and hypertension (Klimes *et al.* 1995). Possible effect of this statin on CYP2C6 expression could present another complicating factor contributing to difficulties in finding the right dose of some drugs metabolized by the human counterpart of this cytochrome P450 (CYP2C9).

MATERIAL AND METHODS

Animals

All procedures with animals were approved by the Ethics Committee, Ministry of Education, Czech Republic. Male Hereditary hypertriglyceridemic Wistar rats (b. w. 250–270 g, seven animals in each group) were maintained under standard conditions. Consequently, the rats were fed *ad libitum* on *a* standard diet (STD), high cholesterol diet (HCD, composed of STD + 1% of cholesterol w/w + 10% of lard fat) and HCD with 0.03% (w/w) of rosuvastatin. The rosuvastatin dose (about 27 mg per kg b.w.) was chosen in agreement with published literature (Galani & Vyas 2010; Reisin *et al.* 2009). The amount of feed consumed was checked daily per each cage holding two animals. After 3 weeks of feeding, the rats were fasted overnight. They were anesthetized by intramuscular administration of fentanyl (40 $\mu g.kg^{-1}$ of body weight) in combination with dexmedetomidin (200 $\mu g.kg^{-1}$ of body weight), followed by administration of diazepam (5 $mg.kg^{-1}$ of body weight). Blood was sampled into EDTA tubes from the aortic bifurcation and plasma was separated by centrifugation (2500 × g, 20 min, 4 °C). Rat liver was removed, rinsed in ice-cold sucrose solution, and frozen in dry ice.

Real-time PCR procedures

A small piece of rat liver tissue sample stabilized in RNAlater (Quiagen, Germantown, MD, USA) was homogenized and subsequently passed through QIAshredder columns to eliminate tissue microparticles. RNA was isolated using RNeasy Plus Minikit (Quiagen Germantown, MD, USA) enabling degradation of contaminating genomic DNA. 1 µg of RNA was reverse-transcribed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) and random hexamer primers. Thusly synthesized cDNA was utilized for RT-PCR using Light Cycler 480 SYBR Green Master I mix in a Light Cycler 480 (Roche, Basel, Switzerland) with the following thermal cycling conditions: 10 min. at 95 °C, followed by 45 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s for denaturation, annealing, and elongation, respectively. All samples for real-time PCR were prepared in triplicates. Cycle-to-cycle fluorescence emission was monitored and absolute quantification method was applied to obtain gene expression data. The respective rat primers were designed in our laboratory and synthesized by Invitrogen (Life Technologies, division Prague, Czech

Republic). The following primer sequences were used:
CYP2C6 Fw 5'-GCCTTGTGGAGGAACTGAGG-3'
CYP2C6 Rev 5'-GCACAGCCCAGGATAAACGT-3'

Western blot analysis

Liver microsomes were obtained using routine procedure as described by Lake (1990). Protein concentration was measured with BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). 12.5 µg of protein from each sample was mixed with buffer (62.5 mM TRIS, 10% glycerol, 4% mercaptoethanol, 2% SDS, pH 6.8) in a volume ratio of 1:1 and electrophoresed on discontinuous SDS-polyacrylamide gel (4% w/w stacking gel and 8% w/w separating gel). Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA). Immunoreactivity was detected by incubation with a primary monoclonal murine CYP2C6 antibody (Abcam, Cambridge, UK) for 60 min and then with a secondary antibody (anti-mouse IgG – HRP conjugate from Sigma, New York, USA). The reaction was detected using enhanced chemiluminescence (ECL) according to the manufacturer's protocol (WB Luminol Reagent, Santa Cruz, CA, USA). The blots were then exposed to medical X-Ray film and scanned using CanoScan Toolbox software, ver. 5.0.

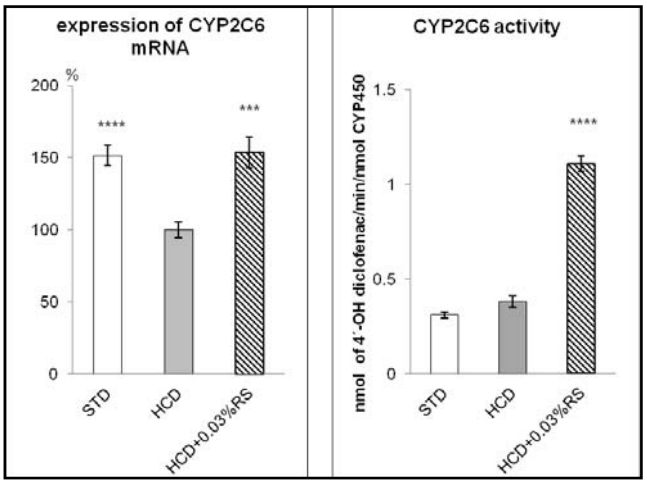

Fig. 1. Effect of standard laboratory diet (STD), high cholesterol diet (HCD) and HCD with rosuvastatin (HCD+0.03%RS) on mRNA expression (A) and activity (B) of CYP2C6. Values are means ± SE, n=7; ***p<0.01, ****p<0.001 *vs* HCD.

Tab. 1. Dietary intakes of standard laboratory diet (STD), high cholesterol diet (HCD) and HCD with 0.03% of rosuvastatin (0.03%RS).

DIET	STD	HCD	HCD+0.03%RS
MDC of diet (g.kg^{-1} body weight)	99.3±1.53	94.0±2.61	91.4±2.53
MDD of drug (mg.kg^{-1} body weight)	–	–	27.4±0.8
Body weight (g)	340±5.1	348±6.3	358±7.1

Values are means ± SE, n=7; MDC – mean daily consumption of the diet, MDD – mean daily dose of rosuvastatin.

Activity of CYP2C6

Formation of selected metabolite of diclofenac (4´-OH diclofenac known to be formed mainly by CYP2C6 (Leemann *et al.* 1993)) as well as the levels of diclofenac (standards obtained from Sigma-Aldrich, Prague, CZ) were determined in samples using a method based on that of Crespi *et al.* (2006). Analyses were performed using HPLC with UV detection set at 280 nm (Shimadzu Prominence System, Kyoto, Japan). The metabolite was separated using a reversed-phase column (Lichrospher 100RP-18, 4.0×250 mm, particle size 5 µm; Merck, Germany) and a C_{18} precolumn (Lichrospher 100, 4×4 mm, 5 µm; Merck, Germany). The column temperature was maintained at 50 °C. Elution of 4'-hydroxydiclofenac was achieved at a flow 1 ml/min. A gradient separation was used with solvent A being 70% (v/v) acetonitrile and solvent B being 100% (v/v) methanol. The gradient steps were as follows: 0–20 min. linear gradient from 70% to 0% solvent A, and from 30% to 100% solvent B; 20–22 min. isocratic at 0% A and 100% B, 22–23 min. gradient from 0% to 70% solvent A, and from 100% to 30% solvent B; 23–33 min. isocratic at 70% solvent A and 30% solvent B.

Statistical analysis

Western blot data were analysed using the ElfoMan software, ver. 2.6 (Semecky Inc., Prague, Czech Republic). All data are expressed as means ± SE, (n=7). Differences between groups were analysed using analysis of variance (ANOVA) followed by the appropriate post-hoc test at the overall significance threshold of p<0.05.

RESULTS

The mean daily consumption of all diets (standard, high-cholesterol, and high-cholesterol with rosuvastatin) was monitored per cage daily during the 21 days of feeding. Mean daily doses of rosuvastatin were calculated from the mean daily consumption of the rosuvastatin-containing diet (at 0.03% of rosuvastatin). The resulting daily dose of rosuvastatin was about 27 mg per kg of body weight. This dose was in agreement with published literature (Reisin *et al.* 2009; Galani & Vyas 2010). Body weights of rats were not significantly affected by any of the experimental diets (Table 1).

Effects of the hypolipidemic drug rosuvastatin on expression of CYP2C6 at the level of mRNA and protein and on hydroxylation activity of this cytochrome P450 in HHTg rats were as follows: rosuvastatin administration (Figure 1) in the high cholesterol and high fat

diet (HCD) lead to a significantly higher expression of CYP2C6 mRNA. Figure 1 also shows the effects of rosuvastatin on hydroxylation activities of CYP2C6 in rats fed the high cholesterol diet. In line with this significant augmentation of mRNA expression, the diclofenac hydroxylating activities corresponding to the CYP2C6 enzyme ((4'-hydroxylation) (Crespi *et al.* 2006)) were also significantly elevated. On the other hand, no significant increase at the level of protein was observed (data not shown).

DISCUSSION

The study presented shows that administration of the hypolipidemic drug rosuvastatin to hereditary hypertriglyceridemic rats fed high cholesterol diet results in an increase in the expression of mRNA of liver microsomal CYP2C6 enzyme, considered to be a counterpart to human CYP2C9 (Daniel *et al.* 2006). This increase has been confirmed by a significant rise of enzymatic activities corresponding to CYP2C6 (diclofenac 4´-hydroxylations (Crespi *et al.* 2006)).

In this study, the sensitivity of CYP2C6 mRNA expression and enzyme activity to rosuvastatin is reported in HHTg rats (an accepted model of metabolic syndrome) for the first time. The mechanism of induction of the CYP2C6 enzyme by statin in rodents with metabolic syndrome has not been elucidated yet.

The *in vitro* inducing profile of rosuvastatin on cytochrome P450 in hepatocytes suggested that this hypolipidemic drug affects the action of nuclear receptors in transcriptional activation of cytochrome P450 genes (Monostory *et al.* 2009). Rosuvastatin was found to increase constitutive androstane receptor-mediated (CAR) transcription of CYP3A4, CYP2C9 (counterpart of CYP2C6 in rat), and CYP2B6 genes in humans (Monostory *et al.* 2009). Moreover, both the effects of high cholesterol diet with high content of fat and the pathological condition of experimental organisms may modulate the final outcome (Orolin *et al.* 2009; Sugatani *et al.* 2010).

Interpretation of our results, obtained for the first time *in vivo*, with regard to possible implications in humans is not straightforward easy. Rat CYP2C6 is not fully identical with human CYP2C9, but exhibits a high sequence identity with this human cytochrome P450 (Daniel *et al.* 2006), which is the form involved in metabolism of many drugs, including S-warfarin (Yamazaki & Shimada 1997). Also, diclofenac is not exclusively the substrate of CYP2C6, it is known to be 4´-hydroxylated also by rat CYP2C11, CYP2D2 and CYP3A1/3A2 (Kobayashi *et al.* 2002, Bruyére *et al.* 2009). On the other hand, Leeman (Leeman *et al.* 1993) found that formation of this metabolite of diclofenac is catalyzed mainly by CYP2C6.

In other words, the fact that the CYP2C6 activity increases significantly whereas with the protein (as estimated from Western blot) a significant increase has not been confirmed may be explained by contribution of other CYP forms to diclofenac 4´-hydroxylation as mentioned above. The estimation of protein expression from Western blot may be also influenced by cross-reactivity of the antibody used (as stated also by the producer, www.abcam.com).

Interactions of rosuvastatin with other drugs are described primarily in terms of an augmented effect of the affected drug following administration of rosuvastatin (Gallelli *et al.* 2009; Galani & Vyas 2010; Pennisi *et al.* 2010). It may be due to an inhibitory effect of statins on drug metabolism by cytochrome P450 or by down regulation of the respective enzyme(s). We reached a similar conclusion in our study using healthy Wistar rats (Zacharová *et al.* 2012). On the other hand, the results obtained here in a rat model of human metabolic syndrome (HHTg rats) indicates that in these rats, i.e. under different conditions, the regulation pathways result in a significantly increased mRNA expression and enzyme activity. This result is in line with previously reported *in vitro* data (Fisslthaler *et al.* 2003; Monostory *et al.* 2009; Feidt *et al.* 2010). These *in vitro* studies were conducted mainly at a cellular level (human hepatocytes, endothelial cells) and not at the level of the whole organism. However, it is unclear the way in which the pathological condition of the experimental organism may affect the mechanism by which rosuvastatin influences the CYP levels and activity. On the other hand, it appears (on the basis of our own results and those published in literature) that rosuvastatin may affect metabolism of many commonly used drugs by influencing the activity and/or expression of cytochrome P450 2C also in man.

CONCLUSIONS

In conclusion, rosuvastatin was found to increase transcription of human CYP2C9 (counterpart of rat CYP2C6) (Monostory *et al.* 2009). In our study rosuvastatin significantly increased mRNA expression and activity of CYP2C6 in HHTg rats. These results are in accordance with previously reported *in vitro* data (Fisslthaler *et al.* 2003; Feidt *et al.* 2010). It may be summarized that rosuvastatin has the potential to influence the levels of drugs metabolized by rat CYP2C6; however, as the relevance of studies performed with experimental models is not straightforward, further studies are needed to elucidate the effects of rosuvastatin on CYP2C9 enzyme in man and also on other pleiotropic pathways (including the effect of pathological state and diet) in humans.

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Potential Conflicts of Interest: *None disclosed.*

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