Master of Science

Correlation of Histological Changes and Nuclear Hormone Receptor Activation in the Rat Liver after Rosuvastatin Calcium Administration

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of the University of Ulsan

Department of Medicine
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Abstract

I designed the *in vivo* study using rats to determine the correlation between activation of nuclear hormone receptor and histologic changes of liver following administration of rosuvastatin calcium. 5-week-old male SD rats were randomly allocated to vehicle treatment group (Control group), rosuvastatin calcium 100 mg/kg treatment group (Low dose treated group; LOW), or rosuvastatin calcium 200 mg/kg treatment group (High dose treated group; HIGH). Rosuvastatin calcium suspended in 0.5 % HPC vehicle or 0.5 % HPC vehicle was administrated orally once a daily, during 7 days. Body weights of HIGH group were decreased since day 3 after first administration and the liver weights of those group were decreased. Serum glucose and cholesterol levels were dose-dependently decreased and serum AST and ALT levels were dose-dependently increased. The histological changes of hepatocyte including cytoplasmic eosinophilic change, anisokaryosis, pknosis or karyorrhexis, and apoptosis were noted in the rosuvastatin calcium treated groups. At immunohistochemical analyses, marked positive immunostaining for CYP4A was observed in the rosuvastatin calcium-treated groups, which was graded dose-dependently. At real-time quantitative PCR analyses, almost CYPs gene levels were dose-dependently decreased following rosuvastatin calcium administration, but Cyp4a levels were not decreased in predominant dose-dependent manner. In conclusion, rosuvastatin calcium-induced histologic changes in this study are likely to be mediated by PPAR α activation, suggesting that these histologic changes are less relevant to human. However, the results in this study are not sufficient to determine whether rosuvastatin calcium is an activator of PPAR α, due to the phenomenon of CYPs depletion or consumption, considered as results of excessive dose treatment. Accordingly, further studies are needed to evaluate peroxisome proliferation, which is induced species-specifically in rodents by activation of PPAR α, or to analyze which nuclear hormone receptor or CYP was activated by rosuvastatin calcium administration.

Keywords: enzyme induction, nuclear hormone receptor, cytochrome P450, rosuvastatin calcium, SD rat

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Introduction

The liver is a multi-lobulated organ located in cranial abdomen and it has a unique blood circulation system which consists of hepatic artery, portal vein, and central vein. The blood is supplied to the liver parenchyma (hepatocyte) via two sources. the major one (75%) is the blood flow exiting splanchnic beds (spleen, pancreas, stomach, small and large intestines) in portal vein and the minor one is abdominal aortic supply (25%) via hepatic artery. Within this microarchitecture of the lobule, the blood flows from portal areas into the sinusoids and out through the central vein. The direction of blood flow across sinusoids accounts for metabolic differences between periportal and centrilobular hepatocytes. Dependent on these natures, the liver is likely to be susceptible to drug-induced injury suggesting hepatotoxicity of drug. Hepatotoxicity has been considered to be major reason for development failure or withdrawal of drug, accordingly, the liver is considered to be one of the important target organs in which toxicity or adverse effects are evaluated to in the preclinical development of drug candidate. [1-5].

Cytochrome P450s (CYPs) are the most important drug-metabolizing enzyme family that play central roles in the phase 1 metabolism (oxidative metabolism), which is essential to excrete xenobiotics from body. Oxidative metabolism is mediated by oxidation, reduction, or hydroxylation. The CYP superfamily is divided into families, such as CYP1, CYP2, CYP3, and CYP4, dependent on identical proportion (>40%), and CYP families were divided into subfamily labelled with letters, dependent on identical proportion (>55%) [6, 7]. CYP 1, 2 and 3 families appear to be responsible for the metabolism of drugs and other xenobiotics, but they are also involved in metabolic conversion of a variety of endogenous compounds such as vitamins, bile acids and hormones. In animals and in human, CYPs could be found in virtually all organs, notably the liver, small intestine, skin, nasal epithelium, lung and kidney, but also in testis, brain and other organs. Especially, the liver and the intestine are the specialized organ for CYP-mediated drug metabolism and elimination whereas the other

organs or tissues contribute to those action to a relatively slight extent [8, 9].

The term 'CYP induction' means the increase in the amount and activity of CYPs following administration of some xenobiotics, or endogenous compounds [10]. This reaction is mediated by some nuclear hormone receptors, including including AhR (Aryl hydrocarbon Receptor), CAR (Consitutive Androstane Receptor), PXR (Pregnane X Receptor), and PPAR α (Peroxisome Proliferator-Activated Receptor α) [11-13]. Because each receptor regulates transcriptional activation of distinct CYP subfamily, analysis for xenobiotics-induced CYPs induction could be performed to determine which nuclear hormone receptor is activated by administration of xenobiotics.

AhR is a one of transcription factor classified as the bHLHPAS (basic helix-loop-helix/Per-Arnt-Sim) gene family [14]. When AhR is activated by some chemicals such as polychlorinated dibenzo(p)dioxins (PCDD), and coplanar polychlorinated biphenyls (PCBs), It is translocated from cytoplasm to nucleus and is subsequently heterodimerized with Arnt (AhR nuclear translocator) known as binding partner of AhR. Ultimately, transcription of target genes is activated. As a potent AhR activator, TCDD is well known to induce multiple drug-metabolizing enzymes, including CYP1A1, CYP1A2, and UGT 1A6 [14]. Also, activation of AhR could induce hepatomegaly characterized by histological changes, such as hepatocyte hypertrophy, multinucleated hepatocytes, fatty change, necrosis, and suppression of apoptosis. Following these histological changes, Eventually, hepatocellular tumor could be formed when AhR activators are chronically exposed [11, 15-17]

CAR is one of the orphan nuclear receptor translocated to nucleus when heterodimerized with PXRa (Retinoic X Receptor), resulting in transactivation of target genes. CAR is characterized by being likely to be activated independent to ligand. Characteristically, CAR is known to be activated independent to ligand [18]. CAR, however, is activated by a diverse range of ligands including 1,4-bis(2-(3,5-dichloropyridoxyloxy)) benzene (TCPOBOP) (murine CAR specific), CICO (human CAR specific), phenobarbital, and clotrimazole (activators of both CAR and PXR) [19-21]. Activated CAR heterodimers bind to a number of

regulatory regions of target genes eliciting transactivation of CYP2B genes.

PXR is well known to regulate transcriptional activation of CYP3A genes. [22, 23]. In addition to CYP3A family members, PXR regulates other genes involving in conjugation, or transport, such as Cyp7a1 (cholesterol 7α-hydroxylase), UGT (UDP-glucuronosyltransferase) 1A1, Mdr1 (multidrug resistance 1; P-glycoprotein), Oatp 2 (organic anion transporting polypeptide 2), and Mrp 2 (multidrug resistance-associated protein) [24-28]. It has been reported that hepatocellular hyperplasia or hypertrophy which are consistent with the response seen at other nuclear hormone receptors are caused by administration of PXR activator, such as PCN (5-Pregnen-3β-ol-20-one-16α-carbonitrile), and CPA (cyproterone acetate). These histological changes are likely to be characterized by increased nuclear ploidy [29]. As with other nuclear hormone receptor activator, it is known that chronic administration of PXR activators results in hepato-carcinogenesis in the rodents [30, 31].

PPAR α a is a major PPAR that is expressed at high levels in liver, kidney, intestine, heart, and brown adipose tissue. PPAR α activator include not only natural ligands such as saturated or unsaturated fatty acids, eicosinoids, and linoleic acid metabolites, but also various xenobiotics such as hypolipidemic agents classified to fibrates (clofibrate, fenofibrate, gemfibrozil, etc.), methaphenilene, thromboxane synthesis inhibitors, dehydroepiandrosterone, ibuprofen, and Wy-14,643 [32]. Activation of PPAR α by fibrate causes liver enlargement by inducing proliferation of peroxisome and sER, as well as CYP4A induction in the rodents [33, 34].

If some histological changes such as hepatocellular hypertrophy, anisokaryosis, and cytoplasmic eosinophilic change of hepatocyte were noted in preclinical toxicity study, it could be important to investigate which nuclear hormone receptor is involved. While it is known that hepatocellular tumor formation by administration of activators of CAR, PXR, or PPAR α is likely to be a rodent-specific response which is few responsive in human, hepato-carcinogenicity by the activation of AhR observed in rodents is concerned as adverse effect

in human [12, 13, 35-37].

Rosuvastatin calcium, a statin class drug developed as an HMG CoA (3-hydroxy-3-methylglutaryl CoA) reductase inhibitor, known to induce morphologic changes of hepatocyte, such as hepatocellular hypertrophy, cytoplasmic eosinophilic change, and karyomegaly in the liver of rats [38, 39]. These toxicity data suggest that rosuvastatin calcium has a potential to be CYP inducer, activating nuclear hormone receptors in rats. However, it is unclear which nuclear hormone receptor is correlated with the histologic changes induced by rosuvastatin calcium administration. accordingly, I designed the *in vivo* study using rats to determine the correlation between activation of nuclear hormone receptor and histologic changes of liver.

Materials and Methods

1. Materials and Chemicals

Rosuvastatin calcium API was supplied by samohpham inc. (Seoul, South Korea), which being manufactured from Teva API India Ltd. (Gajraula, India) and Hydroxypropyl cellulose (HPC) powder used as vehicle material was purchased from Sigma-Aldrich (Cat no. 435007, St. Louis, Missouri). All reagents available with BenchMark XT (Ventana Medical Systems, Tucson, AZ), automated immunohistochemistry stainer, were purchased from inseonmedics inc. (Seoul, South Korea), except for antibody diluent(Cat. No. S0809, Dako inc., Kyoto, Japan).

2. Experimental Design

Approximately 5-week-old, SPF (Specific Pathogen Free) male SD rats were obtained from Orient Bio (Gyeonggi-do, Korea) and a total of 30 rats were used. Each rats were randomly allocated to vehicle treatment group (Control group), rosuvastatin calcium 100 mg/kg treatment group (Low dose treated group; LOW), or rosuvastatin calcium 200 mg/kg treatment group (High dose treated group; HIGH), in which 10 rats were assigned per group (table 1). 2 or 3 rats were housed per polysulfone cage with a wire mesh roof and maintained on a certified rodent diet manufactured by purina inc. (St. Louis, Mo.), under conditions of temperature, relative humidity, ventilation, and illumination. The study protocol was reviewed and approved by the Animal Care and Use Committee of the Asan Medical Center (IACUC No. 2018-12-096).

Table 1. Experimental design.

In vivo rats 1-week repeated dose administration study				
Group	No. of rats	Dose (mg/kg)	Administration volume (ml/kg)	Route of administration
Control	10	0	10	Oral administration
LOW	10	100	10	Oral administration
HIGH	10	200	10	Oral administration

All rats were acclimatized for 7 days, followed by administration phase. Rosuvastatin calcium suspended in 0.5 % HPC vehicle or 0.5 % HPC vehicle was administrated orally once a daily, during 7 days. One day after the last administration, measurement of body weight was performed, subsequently all rats were anesthetized using isoflurane and blood was collected in Becton Dickinson Vacutainer 2ml SST tubes from caudal vena cava for serum biochemistry analyses. Then euthanasia was progressed through exsanguination of the caudal vena cava, followed by measurement of liver weights. Subsequently partial of left lateral lobe was collected in cryotube for real-time PCR analyses and maintained at -70 ℃ *until analysis performed*, and remained liver tissue was collected in 10% neutral buffered formalin (NBF) for histopathological examination.

3. Serum Biochemistry

All blood samples in SST tube were maintained during 2 hours at room temperature, and subsequently centrifuged at 2000 rpm for 10 minutes to acquire serum samples. Serum AST (Aspartate aminotransferase), and ALT (Alanine aminotransferase) levels were measured to evaluate severity of liver injury by using an automated clinical chemistry analyzer (Hitachi 7180; Hitachi High-Technologies, Japan).

4. Histopathological Examination

All liver tissue fixed in 10% Neutral Buffered Formalin (10% NBF) were dehydrated in graded ethanol, cleared in xylen using a Shandon Excelsior ES tissue processer (Thermo Fisher Scientific), and embedded in paraffin block using an EG1150H paraffin-embedding station (Leica Biosystems, Wetzlar, Germany). Paraffin-embedded blocks were sectioned at a 4 μm thickness and mounted on slide glass, followed by Hematoxylin and Eosin (H&E) staining. Histopathological examination of the liver was performed by an experienced veterinary pathologist (Woo-Chan Son, Asan Medical Center, Korea). For quantitative evaluation of hepatocellular karyomegaly, nuclear diameters of 10 hepatocytes were measured at 400X magnification field, a total of six 400X magnification fields per animal, using image analysis program. Subsequently, the mean nuclear diameter was calculated.

5. Immunohistochemistry

To evaluate translocation of nuclear hormone receptors (AhR, CAR, PXR, and PPAR α) from cytoplasm to nucleus and expression of CYP450 enzymes (CYP1A, CYP2B, CYP3A, and CYP4A Subfamily), immunohistochemistry was performed. Paraffin-embedded block of SD rat liver tissue were cut at a 4 μm thickness and sections were mounted on coated glass slides. After deparaffinization, antigen retrieval and immunostaining were performed using using Cell Conditioning Solution (CC1) and Ultraview DAB detection kit (Ventana Medical Systems, Inc, Tucson, AZ) on a Ventana BenchMark XT (Ventana Medical Systems Inc., Tucson, AZ) according to the manufacturer's protocol. List of the primary antibodies were as follow (table 2), and UltraMap Anti-Ms/Rb HRP(Ventana Medical Systems Inc., Tucson, AZ) was used as secondary antibody. Immuno-stained sections were counterstained with hematoxylin and bluing reagent, and cover-slip was mounted using lipid-soluble mountant. Degree of the translocation of nuclear hormone receptors was semi-quantitatively graded as negative (0), or positive (+1). The intensity of positive staining for CYP enzymes was semi-quantitatively graded as minimal (+1), slight (+2), moderate (+3), marked (+4) or severe (+5).

Table 2. Primary antibodies used for immunohistochemistry

Antibody	Cat no.	Titration	Incubation time	Host	Type	Manufacturer
Anti-CYP1A1/1A2	MA3-036	1:200	36 min	Mouse	Monoclonal	ThermoFisher Scientific, San Jose, CA, USA
Anti-CYP2B1/2B2	sc-73546	1:400	36 min	Mouse	Monoclonal	Santacruz biotechnology, California, USA
Anti-CYP3A4	ab3572	1:400	36 min	Rabbit	Polyclonal	Abcam inc.,
Anti-CYP4A	ab3573	1:1000	36 min	Rabbit	Polyclonal	Abcam inc.,

6. RNA Extraction and Reverse Transcription

At necropsy, a portion of left lobe of liver was separately collected and placed in cryotube before liver was fixed in 10% neutral buffered formalin. The cryotubes were immediately preserved in liquid nitrogen, subsequently were moved to deep freezer and preserved at -80 ℃ within 2 hours after collection. Total RNA was extracted from liver collected from all groups by the GeneAll$_R$ Hybrid-R$_{TM}$ RNA extraction kit (Cat. No. 305-101, GeneAll Biotechnology, Republic of Korea), according to manufacturer's instructions. Total RNA levels in the final eluent were determined using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Then the first-strand complementary DNA (cDNA) was synthesized using RevertAid$_{TM}$ First Strand cDNA Synthesis Kit (K1622, Thermo Scientific, Wilmington, DE, USA), according to manufacturer's instructions.

7. Real-time Quantitative PCR (RT-qPCR)

Approximately 4 µl of total cDNA was used for the RT-PCR, in a 20 µL reaction mixture comprised of 0.4 µl of stock solution for forward and reverse primers suitable for target genes, 10 µL of 2X GoTaq® qPCR Master Mix, and 0.4 µl CXR (Promega A6001/2, USA). All primers were manufactured by Cosmo Gentech Co. (Seoul, Korea). Sequences of the

primers are as follows (table 3). The relative mRNA levels of nuclear hormone receptors and cytochrome P450 enzymes was calculated using $2^{-\Delta\Delta CT}$ method [40]. As housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as previous described.

Table 3. The primer used for RT-qPCR

Gene	Sequence of forward primer	Sequence of forward primer	Gene bank accession no.
Cyp1a1	GCT GAG GCT CAA CTG TCT TCC A	GGC TTT GCA AGG ACAAGG AGA	NM_012540.2
Cyp1a2	AGG AGA TGC TCA ACC TCG TG	AGA AGT CCA CAG CAT TCC CTG	NM_012541.3
Cyp2b1	TGA GAA CCT CAT GAT CTC CCT GC	AGG AAA CCA TAG CGG AGT GTG G	NM_001134844.1
Cyp2b2	GTC GAA CCA CCA CAC AGA GT	TCA TCA AGG GAT GGT GGC CT	NM_001198676.1
Cyp3a2	GGA GTT GGC AAG GTC TGT GA	GAT GTG GAT GGA GAT GGT CCC	NM_153312.2
Cyp3a9	CCT GGC TGC TCC TGGTTA TC	GGC CCA GGA ATT CCCAAC TT	NM_147206.2
Cyp4a1	CTG CTC CGC TTT GAG CTA CT	CGG AGC TCC ACA ACG GAA TTA	NM_175837.1
Cyp4a3	AAG AAT GGG ATC CAC CTG CG	TAT TGC AGG CAG CAG ACC TC	NM_175760.2
GAPDH	AGT GCC AGC CTC GTC TCA TA	GGT AAC CAG GCG TCC GAT AC	NM_017008.4

8. Statistical Analyses

All ordinary values are expressed as the mean ± standard error of the mean. ANOVA or Kruskal-Willis test were performed for multiple comparisons by using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). P-value < 0.5 was considered statistically significant.

Results

1. Body and Liver Weights

Body weights was recorded at day 1 (first administration), 3, 5, 7, and necropsy (day 8) per group (Figure 1). Body weight in the HIGH group gradually decreased since day 3 after first administration and mean of body weights in the HIGH group were statistically significantly lower than those in the control group at day 5 (*P-value* < 0.001), day 7 (*P-value* < 0.0001), and day of necropsy (*P-value* < 0.0001). At necropsy, the absolute liver weights were recorded and the relative liver weights were calculated by dividing the absolute liver weight by the body weight (Figure 2A, B). There was statistically significant decrease of the absolute liver weight (*P-value* < 0.001) and the relative liver weight (*P-value* < 0.05) in the HIGH group, compared with control group.

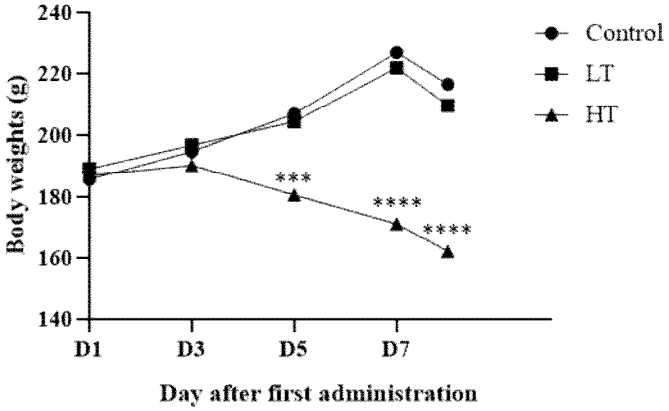

Figure 1. Time-course changes in mean body weights during administration period. Since day 5, body weights of HIGH group were lower than those of control group.

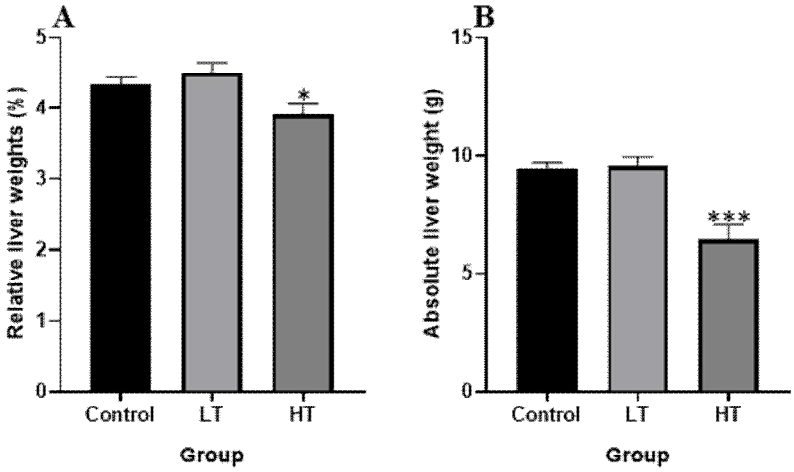

Figure 2. The absolute and relative liver weights. there were statistical significances of both parameters in HIGH group.

2. Serum Biomarker Levels

The serum glucose, cholesterol, AST, and ALT levels were measured in serum collected at necropsy (Figure 3). The mean levels of serum glucose and cholesterol were dose-dependently decreased. The mean serum glucose level was statistically significantly decreased in the HIGH group (*P-value* < 0.0001), and the mean serum cholesterol levels were statistically significantly decreased in the both rosuvastatin calcium-treated groups (*P-value* < 0.05, *P-value* < 0.01, respectively).

The mean serum AST levels were 2-fold and 7-fold higher in the LOW and HIGH group, respectively, when compared to those of the control group. The levels of this enzyme in the HIGH group was statistically significantly higher than those in the control group (*P-value* < 0.01). Also, the mean serum ALT levels were 2-fold and 5-fold higher in the rosuvastatin calcium-treated group, respectively, when compared to those of the control group. The levels of this enzyme in the HIGH group was statistically significantly higher than those in the control group (*P-value* < 0.05).

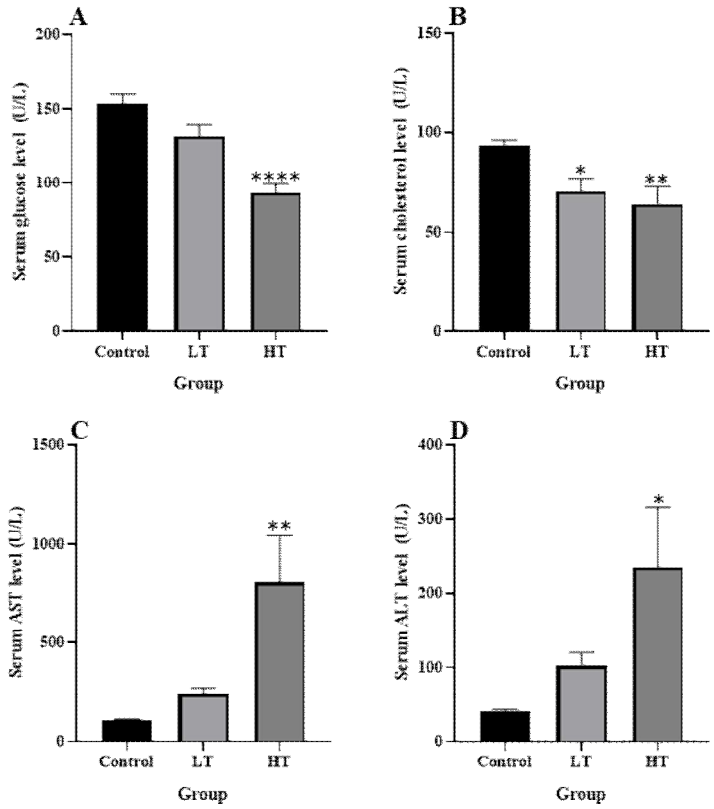

Figure 3. Serum glucose (A), cholesterol (B), AST (C), and ALT (D) levels. Serum glucose and cholesterol levels were dose-dependently decreased. Serum AST and ALT levels were dose-dependently increased and those levels in the HIGH group was statistically significantly increased.

3. Histopathological Examination of The Liver

Some histopathologic findings of hepatocyte were observed, including cytoplasmic eosinophilic change, anisokaryosis, karyorrhexis, or pyknosis, in the rosuvastatin calcium-treated rats. pyknotic hepatocyte was characterized by chromatic condensation in the periphery nuclei with irregular nuclear membrane. In addition to alterations of nuclei, Apoptotic bodies were noted with eosinophilic cytoplasm was also increased in the rosuvastatin calcium-treated rats.

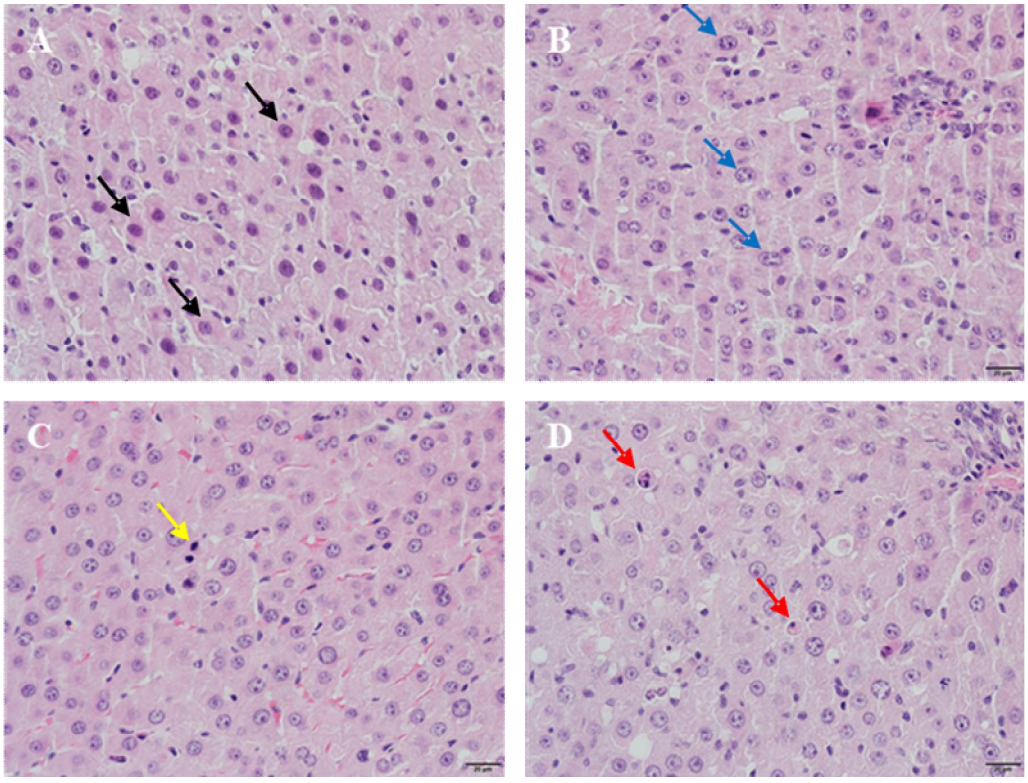

Figure 4. Microscopic examination of the liver. There were some histopathological findings including cytoplasmic eosinophilic change (Figure 4A, black arrow), anisokaryosis (Figure 4B, blue arrow), pyknosis (Figure 4C, yellow arrow), and increase in apoptosis of hepatocyte (Figure 4D, red arrow) in SD rats treated by rosuvastatin calcium. H&E stain, 400X magnification, Scale bar = 20 μm.

4. Immunohistochemical Expression of CYP1A, CYP2B, CYP3A, and CYP4A Subfamily

Mean of intensity score for immunohistochemical expression of CYPs are shown in Table 4. Positive immunostaining for CYP1A1/1A2 of control group was characterized by focally clustered cytoplasmic staining (Figure 5.A), while those of rosuvastatin calcium treated group were noted as diffuse cytoplasmic pattern with low intensity (Figure 5.B). The mean of intensity score for CYP1A1/1A2 were dose-dependently decreased in the LOW and HIGH groups compared to the control group, which were 1.6 and 1.5, respectively. However, the difference in staining intensity was minimal, and was equivocal. Like the results of CYP1A1/1A2, the score of positive staining for CYP2B1/2B2 were dose-dependently decreased, which were 1.7 and 1.2 in the LOW and HIGH groups, respectively. but the difference in positive staining intensity was macroscopically significantly noted (Figure 5.C, D). There were no significant differences in positive staining for CYP3A4 between control and Rosuvastatin calcium-treated groups, in which almost slides were graded as score 1 or 2 (Figure 5.E, F). In the rosuvastatin calcium-treated rats, Marked positive immunostaining for CYP4A was noted diffusely at the hepatocytic cytoplasm (Figure 5.H). The mean of intensity score for CYP4A dose-dependently increased, which were 3.1 and 3.6 in the LOW and HIGH groups, respectively. Positive immunostaining for CYP4A in the HIGH group was characterized to spread from the centrilobular area (zone 3) to the periportal area (zone 1).

Table 4. Semi-quantitative score of CYPs levels at immunohistochemistry

Group	CYP1A1/1A2	CYP2B1/2B2	CYP3A4	CYP4A
Control	1.9 ± 0.18	2.9 ± 0.18	1.6 ± 0.22	2.6 ± 0.22
LOW	1.6 ± 0.22	1.7 ± 0.26	1.6 ± 0.16	3.1 ± 0.28
HIGH	1.5 ± 0.22	1.2 ± 0.13	1.7 ± 0.26	3.6 ± 0.22

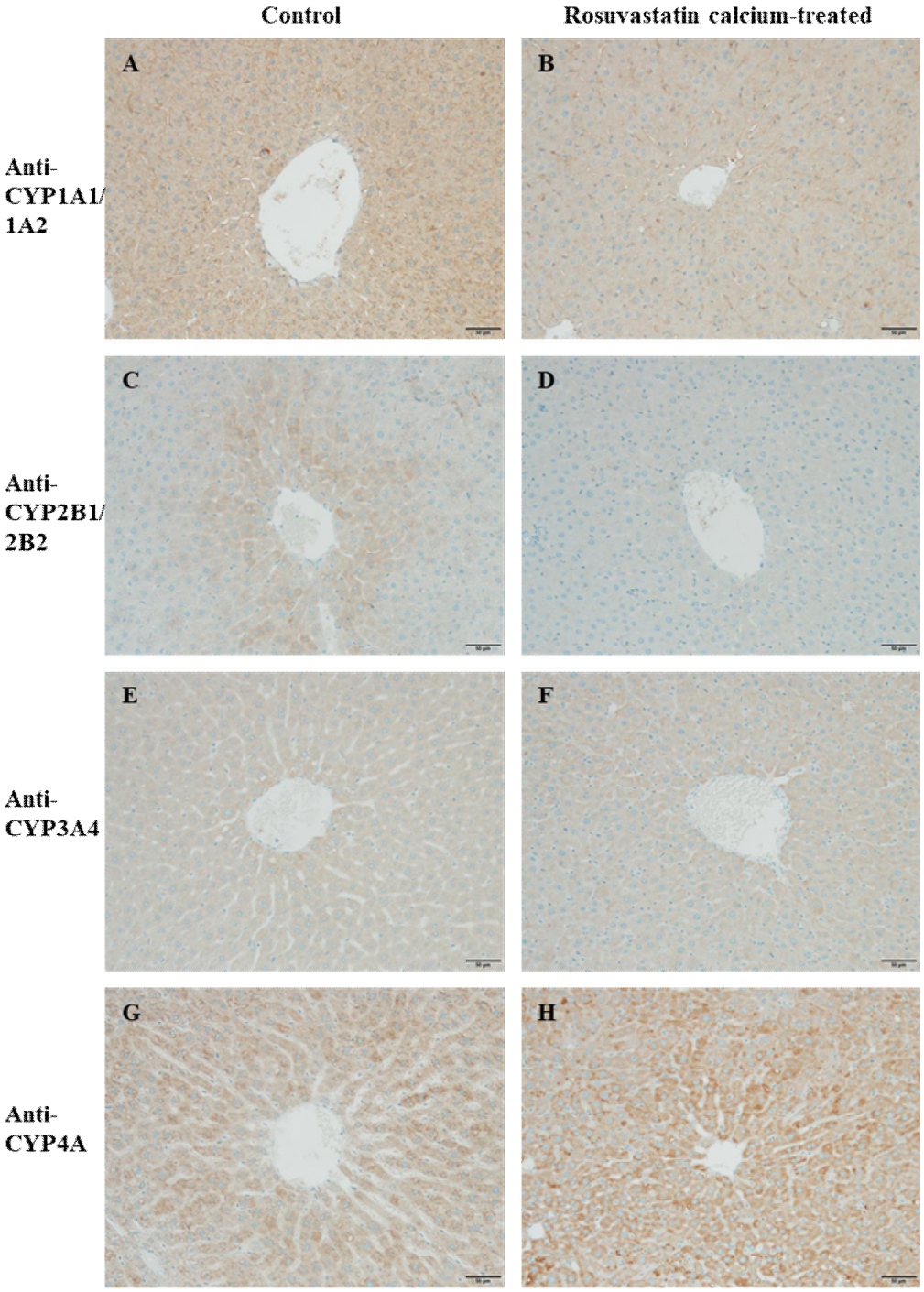

Figure 5. Immunohistochemistry for CYP1A1/1A2 (Figure 5A, B), CYP2B1/2B2 (Figure 5C, D), CYP3A (Figure 5E, F) and CYP4A (Figure 5G, H). formalin-fixed, paraffin-embedded sections, 200X magnification, Scale bar = 50 µm.

6. mRNA Levels of Cyp1a, Cyp2b, Cyp3a, and Cyp4a

To evaluate effect of rosuvastatin calcium on the induction of CYPs (Cyp1a, Cyp2b, Cyp3a, and Cyp4a) respectively mediated by AhR, CAR, PXR, and PPAR α, mRNA levels of those CYPs were also analyzed. While there were no statistically significant changes of Cyp1a1 mRNA levels, mRNA levels of Cyp1a2 in the rosuvastatin calcium-treated groups were decreased dose-dependently. In addition, those in the HIGH group were statistically significantly lower (*P-value* < 0.01) than those of control group (Figure 6.A, B). mRNA levels of Cyp2b1 and Cyp2b2 were dose-dependently decreased and statistical significances were noted in the HIGH group (*P-value* < 0.05, Figure 6.C) when comparing those of control group. Like those of Cyp2b1, mRNA levels of Cyp2b2 were decreased in dose-dependent manner, there were also statistically significant difference between the control and HIGH group (*P-value* < 0.05, Figure 6.D). mRNA levels of Cyp3a2 and Cyp3a9 were also dose-dependently decreased (Figure 6.E, F), and the statistical significances were noted in the HIGH (*P-value* < 0.05) group at the analyses for both Cyp3a genes. In contrast, no mRNA levels of Cyp4a1 and Cyp4a3 genes were changed in predominant dose-dependent manner (Figure 6.G, H).

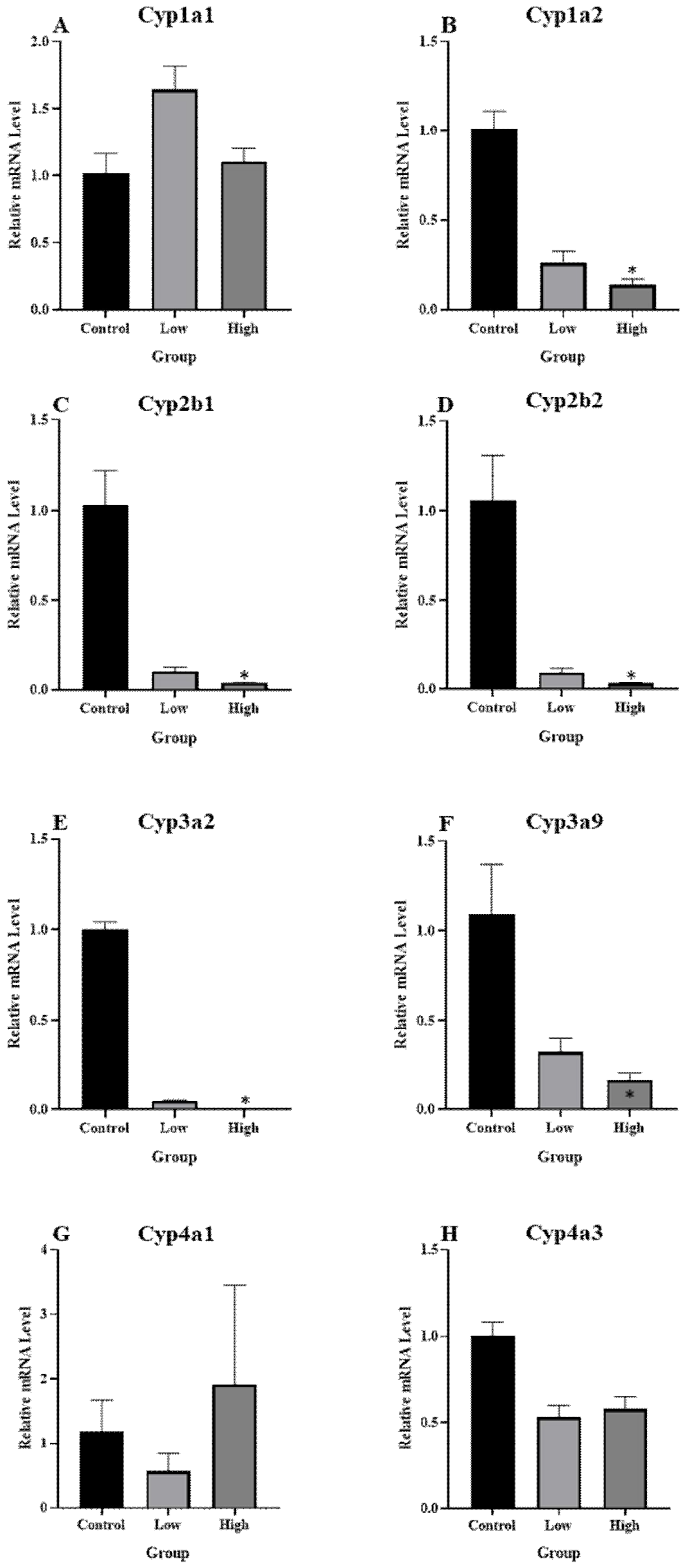

Figure 6. mRNA levels of Cyp1a (Figure 6.A, B), Cyp2b (Figure 6.C, D), Cyp3a (Figure 6.E, F), and Cyp4a (Figure 6. G, H)

Discussion

It has been noted that the histological changes, which are associated with enzyme induction (hepatocellular hypertrophy, anisokaryosis, cytoplasmic eosinophilic change of hepatocyte, etc.), is induced by administration of rosuvastatin calcium in rats [38, 39]. However, it is not well known whether these histological changes are mediated by activation of nuclear hormone receptors (AhR, CAR, PXR, or PPAR α). So, to investigate the correlation between hepatic histological changes and nuclear hormone receptor activation, i performed short-term repeated dose toxicity study in rats, and subsequently analyzed expression levels of CYP subfamilies (CYP1A, CYP2B, CYP3A, and CYP4A).

First, I confirmed that the effect of rosuvastatin calcium on body weights. From day 3 after first administration, body weights in the HIGH group were statistically significantly decreased, in which mean body weights of HIGH group were decreased up to 75 % of those of control group. These changes are thought to have occurred due to the excessive pharmacological action of the rosuvastatin calcium, which is thought to be correlated with decreased serum glucose and cholesterol levels.

The absolute, or relative liver weights was statistically significantly lower in the HIGH group, comparing to control group. Hepatic atrophy can be mainly caused by severe caloric deficits, and exposure to high doses of xenobiotics. Accordingly, it is suggested that hepatic atrophy occurred by the administration of excess high dose of rosuvastatin calcium, although hepatocellular atrophy was not detected in histopathologic examination.

In the results of histopathological examination, some histopathologic findings of hepatocyte were observed, including presents of hepatocytes with eosinophilic cytoplasm, anisokaryosis, apoptosis, and karyorrhexis or pyknosis in the rosuvastatin calcium-treated groups. It is well known that hepatic hypertrophy is commonly associated with microsomal enzyme induction secondary to exposure to certain xenobiotics in laboratory animals [41-44], however, which was not detected in the rosuvastatin calcium-treated rats in this study.

Cytoplasmic eosinophilic change of hepatocyte in this study suggests that administration of rosuvastatin calcium induce CYPs, although hepatocellular hypertrophy was not detected. This result is thought to occurred due to the administration of excessive dose for a very short period.

There were unexpected results at immunohistochemical and RT-qPCR analyses, which was that analyzed CYPs except for CYP4A tend to decrease following administration of rosuvastatin calcium. This tendency is thought to be associated with atrophic change of liver following the treatment of excessive dose of rosuvastatin calcium, and it is suggested that excessive treatment of rosuvastatin calcium induce depletion or consumption of diverse CYPs from rosuvastatin calcium-metabolizing enzymes to CYPs which are not involved in metabolism of rosuvastatin calcium. Therefore, uncertainty is remained as to which CYPs is induced by treatment of rosuvastatin calcium. However, when considering the results of analyses for CYP4A levels, it is thought to be important to consider the possibility that CYP4A subfamily is induced by administration of rosuvastatin calcium. At results of immunohistochemical analyses, the significant difference of immunostaining intensity for CYP4A was shown distinctly unlike results for other CYPs whose intensity decreased or was maintained when compared to those of the control group. mRNA level of Cyp4a was lower than that of the control group, which is presumably due to an increase in the consumption of almost CYPs due to excessive dose treatment. However, Cyp4a mRNA levels of the high dose treated group was higher than those of the low dose group, and degree of decrease in Cyp4a mRNA levels was weak relative to other Cyp genes. therefore, it is suggested that rosuvastatin calcium induce CYP4A subfamily in rats, and that CYPs induction which is not accompanied by hepatic hypertrophy could occur in the preclinical short-term toxicity study, although hepatic hypertrophy is considered as typical hallmark to CYPs induction. Accordingly, when based on interpretation for results in this study, it is emphasized that even if no drug-induced hepatic hypertrophy is present in preclinical short-term toxicity study, it should not be rashly judged that CYPs induction has not occurred.

In conclusion, rosuvastatin calcium-induced histologic changes in this study are likely to be mediated by PPAR α activation, suggesting that these changes are less relevant to human. However, the results in this study are not sufficient to determine whether rosuvastatin calcium is an activator of PPAR α. Therefore, further studies are needed to evaluate peroxisome proliferation, which is induced species-specifically in rodents by activation of PPAR α, or to analyze which nuclear hormone receptor or CYP was activated by rosuvastatin calcium administration.

References

1. Thoolen, B., et al., *Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system.* 2010. **38**(7_suppl): p. 5S-81S.
2. Ballet, F.J.J.o.h., *Hepatotoxicity in drug development: detection, significance and solutions.* 1997. **26**: p. 26-36.
3. Cattley, R.C. and J.M. Cullen, *Liver and gall bladder*, in *Haschek and Rousseaux's Handbook of Toxicologic Pathology (Third Edition)*. 2013, Elsevier. p. 1509-1566.
4. Haschek, W.M., et al., *Haschek and Rousseaux's handbook of toxicologic pathology*. 2013: Academic Press.
5. Chen, M., et al., *FDA-approved drug labeling for the study of drug-induced liver injury.* 2011. **16**(15-16): p. 697-703.
6. Nelson, D.R., et al., *The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature.* 1993. **12**(1): p. 1-51.
7. Gonzalez, F.J.P.r., *The molecular biology of cytochrome P450s.* 1988. **40**(4): p. 243-288.
8. Peters, W.H. and P.G.J.B.p. Kremers, *Cytochromes P-450 in the intestinal mucosa of man.* 1989. **38**(9): p. 1535-1538.
9. Shimada, T., et al., *Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians.* 1994. **270**(1): p. 414-423.
10. Pelkonen, O., et al., *Inhibition and induction of human cytochrome P450 (CYP) enzymes.* 1998. **28**(12): p. 1203-1253.
11. Maronpot, R.R., et al., *Hepatic enzyme induction: histopathology.* 2010.

38(5): p. 776-795.

12. Hall, A., et al., *Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes—conclusions from the 3rd International ESTP Expert Workshop.* 2012. **40**(7): p. 971-994.
13. Tompkins, L.M., A.D.J.J.o.b. Wallace, and m. toxicology, *Mechanisms of cytochrome P450 induction.* 2007. **21**(4): p. 176-181.
14. Schwarz, M., K.E.J.R.T. Appel, and Pharmacology, *Carcinogenic risks of dioxin: mechanistic considerations.* 2005. **43**(1): p. 19-34.
15. Bock, K.W. and C.J.B.p. Köhle, *Ah receptor-and TCDD-mediated liver tumor promotion: clonal selection and expansion of cells evading growth arrest and apoptosis.* 2005. **69**(10): p. 1403-1408.
16. Kasai, T., et al., *Two-year inhalation study of carcinogenicity and chronic toxicity of 1, 4-dioxane in male rats.* 2009. **21**(11): p. 889-897.
17. Yoshizawa, K., et al., *A critical comparison of murine pathology and epidemiological data of TCDD, PCB126, and PeCDF.* 2007. **35**(7): p. 865-879.
18. Honkakoski, P. and M.J.B.J. Negishi, *Regulation of cytochrome (CYP) genes by nuclear receptors.* 1998. **347**: p. 321-37.
19. Wei, P., et al., *The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism.* 2000. **407**(6806): p. 920.
20. Maglich, J.M., et al., *Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes.* 2003. **278**(19): p. 17277-17283.
21. Moore, L.B., et al., *Orphan nuclear receptors CAR and PXR share xenobiotic and steroid ligands.* 2000.
22. Bertilsson, G., et al., *Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction.* 1998. **95**(21): p. 12208-12213.

23. Gibson, G.G., W. El-Sankary, and N.J.J.T. Plant, *Receptor-dependent regulation of the CYP3A4 gene.* 2002. **181**: p. 199-202.

24. Wang, H., et al., *Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes.* 2003. **31**(5): p. 620-630.

25. Staudinger, J., et al., *Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor.* 2001. **29**(11): p. 1467-1472.

26. Hartley, D.P., et al., *Activators of the rat pregnane X receptor differentially modulate hepatic and intestinal gene expression.* 2004. **65**(5): p. 1159-1171.

27. Geick, A., M. Eichelbaum, and O.J.J.o.B.C. Burk, *Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin.* 2001. **276**(18): p. 14581-14587.

28. Fromm, M.F., et al., *The effect of rifampin treatment on intestinal expression of human MRP transporters.* 2000. **157**(5): p. 1575-1580.

29. Schulte-Hermann, R.J.C.c.r.i.t., *Induction of liver growth by xenobiotic compounds and other stimuli.* 1974. **3**(1): p. 97-158.

30. Schuppler, J. and P. Günzel, *Liver tumors and steroid hormones in rats and mice*, in *Mechanism of Toxic Action on Some Target Organs*. 1979, Springer. p. 181-195.

31. Tucker, M., A. Kalinowski, and T.J.C. Orton, *CARCINOGENESIS: Carcinogenicity of cyproterone acetate in the mouse.* 1996. **17**(7): p. 1473-1476.

32. Greaves, P.J.H.o.p.t.s., *Liver and pancreas.* 2007: p. 457-503.

33. Cattley, R.C.J.M.r. and technique, *Regulation of cell proliferation and cell death by peroxisome proliferators.* 2003. **61**(2): p. 179-184.

34. Walker, R.M., et al., *Hepatotumorigenicity and peroxisome proliferation induced by the hypolipidemic CI-924 in a two-year study in male and female*

B6C3F1 mice. 1996. **24**(3): p. 265-272.

35. Martignoni, M., et al., *Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction.* 2006. **2**(6): p. 875-894.

36. Graham, M.J. and B.G.J.T. Lake, *Induction of drug metabolism: species differences and toxicological relevance.* 2008. **254**(3): p. 184-191.

37. Lake, B.G.J.T.R., *Human relevance of rodent liver tumour formation by constitutive androstane receptor (CAR) activators.* 2018.

38. Al-Doaiss, A.A., S.A. Alarifi, and B.M.J.P.J.o.Z. Jarrar, *Hepatic histological and histochemical alterations induced by rosuvastatin therapeutic doses.* 2013. **45**(1).

39. *Crester Pharmacology Review(s) part 4*. 2003, U.S. Food and Drug Administration.

40. Rao, X., et al., *An improvement of the 2ˆ (–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis.* 2013. **3**(3): p. 71.

41. Amacher, D., S. Schomaker, and J. Burkhardt, *The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies.* Food and chemical toxicology, 1998. **36**(9-10): p. 831-839.

42. Amacher, D., S. Schomaker, and J. Burkhardt, *The relationship among microsomal enzyme induction, liver weight and histological change in beagle dog toxicology studies.* Food and chemical toxicology, 2001. **39**(8): p. 817-825.

43. Amacher, D.E., et al., *The relationship among microsomal enzyme induction, liver weight, and histological change in cynomolgus monkey toxicology studies.* Food and chemical toxicology, 2006. **44**(4): p. 528-537.

44. Ennulat, D., et al., *Diagnostic performance of traditional hepatobiliary*

biomarkers of drug-induced liver injury in the rat. Toxicological sciences, 2010. **116**(2): p. 397-412.

Summary in Korean

본 연구는 SD 랫드 간에서 rosuvastatin calcium 이 간세포 비대(centrilobular hypertrophy)와 관련된 CYP 효소 (CYP1A, CYP2B, CYP3A, 그리고 CYP4A) 중 어떤 CYP 효소를 유도하는지 규명하고자 진행되었다. 시험군은 대조군(0.5% hydroxypropyl cellulose 부형제 투여군), rosuvastatin calcium 100 mg/kg 투여군(저용량군) 및 200mg/kg 투여군(고용량군) 총 3 군으로 설정하였으며 총 30 마리의 SD 랫드를 군 당 10 마리씩 3 군으로 임의적으로 배정하였다. 7 일 간 경구 반복투여를 실시하였으며 마지막 투여일로부터 하루 뒤 부검을 실시하여 체중 및 간 중량을 측정하였고 혈청, 간 검체를 수집하였다. 혈청 생화학 검사를 실시하여 혈청 검체의 혈청 아스파르테이트 아미노전달효소(AST) 및 알라닌 아미노전달효소(ALT) 수치를 측정하였고 간에 대한 조직병리학적 검사를 수행하였다. 핵 호르몬 수용체인 AhR, CAR, PXR, 그리고 PPAR α 활성에 따라 유도되는 각각의 CYP 효소들(CYP 1A, CYP2B, CYP3A, 그리고 CYP4A)에 대한 immunohistochemistry (IHC) 분석을 실시하였다. real-time quantitative PCR (RT-qPCR)분석을 실시하여 CYP 1A, CYP2B, CYP3A, 그리고 CYP4A 의 mRNA 수준을 분석하였다. 실험 결과, 절대적, 그리고 상대적 간 중량이 고용량 투여군에서 통계학적으로 유의하게 감소하였다. rosuvastatin calcium 투여군에서 혈청 glucose 및 cholesterol 이 통계적으로 유의하게 증가하였고 AST 및 ALT 수치가 통계학적으로 유의하게 증가하였다. 조직병리학적 검사 결과, rosuvastatin calcium 투여군에서 간세포 비대 소견은 관찰되지 않았지만, 효소 유도와 관련 있을 수 있는 간세포의 핵 대소부동증(anisokaryosis) 및 세포질의 호산성 변화 (cytoplasmic eosinophilic change) 소견이 유의하게 확인되었으며 추가적으로 핵응축(pyknosis) 또는 핵붕괴(karyorrhexis), 그리고 세포자멸사(apoptosis) 소견이 동반되었다. IHC 분석 결과, rosuvastatin calcium 투여군 간에서 CYP1A, 그리고 CYP2B 에 대한 양성 염색 정도가 용량 의존적으로 감소한 반면에 CYP4A 에 대한 양성 염색 정도는 용량 의존적으로 증가하였다. RT-qPCR 분석 결과, rosuvastatin calcium 투여군에서 CYP1A2, CYP2B1, CYP2B2, CYP3A2, CYP3A9 의 mRNA 수준이 용량의존적으로 감소함과 동시에 통계적으로 유의한 차이가 확인되었지만, CYP1A1, CYP4A1,

CYP4A3 의 mRNA 수준의 경우 뚜렷한 경향이 관찰되지 않았으며 군 간의 통계적인 유의한 차이는 확인되지 않았다. 본 연구에서 Rosuvastatin calcium 투여에 따라 나타난 혈청 AST, ALT 증가 및 간세포 세포질의 호산성 변화, 그리고 간세포 핵의 대소부동증 소견은 간세포 비대와 함께 효소 유도와 함께 동반되어 나타날 가능성이 높은 병변으로, 이에 따라 rosuvastatin calcium 이 SD 랫드 간에서 효소 유도를 일으키는 것을 시사한다. IHC, RT-qPCR 분석 결과를 종합적으로 고려한 결과, rosuvastatin calcium 이 랫드에서 PPAR α 을 직접적으로 활성화시켜 CYP4A 유도에 관여한 것으로 사료된다. 결론적으로, 랫드 간에서의 rosuvastatin calcium 투여에 따른 조직학적 변화들은 PPAR α 활성과 상관관계가 있는 변화인 것으로 판단되며, 이에 따라 본 조직학적 소견들이 설치류 특이적이고, 사람과의 연관성이 매우 낮은 변화일 가능성이 시사된다. 간 중량 감소 및 혈청 glucose 수치 감소 소견과 함께, IHC 와 RT-qPCR 분석에서 확인된 CYPs 단백질, 유전자 수준의 용량 의존적인 감소는 예상하지 못한 결과로서, 단기간 동안 과도한 용량의 rosuvastatin calcium 투여에 의해 간에서 대사 작용이 급격하게 활성화되고 이에 따라 에너지원, 그리고 대사 효소들의 과도한 소비가 유도되어 나타난 결과로 여겨진다. 간세포 비대 소견의 부재 또한 동일한 이유로 발생했을 가능성이 있는 것으로 판단된다. 이러한 판단 하에, 약물을 단기간 동안 고용량으로 처치하는 경우 핵 호르몬 수용체 활성에 따른 효소 유도가 간세포 비대 소견을 동반하지 않고 발생할 가능성이 시사되며, 신약 개발 초기에 단기 독성 시험 수행 시 간세포 비대 소견에 절대적으로 의존하여 효소 유도 및 핵 호르몬 수용체 활성 여부를 판단하는 일은 지양해야 할 것임이 강조된다.