Sterol 27-hydroxylase gene dosage and the antiatherosclerotic effect of Rifampicin in mice

Line Zurkinden¹, Dmitri Sviridov², Bruno Vogt¹ and Geneviève Escher¹

¹Department of Nephrology, Hypertension and Clinical Pharmacology, Department of Clinical Research, Inselspital, University of Bern, Switzerland; ²Baker IDI Heart and Diabetes Institute, PO Box 6492, Melbourne, VIC 3004, Australia

Correspondence: Geneviève Escher (genevieve.escher@dbmr.unibe.ch)

Sterol 27-hydroxylase (CYP27A1) catalyzes the hydroxylation of cholesterol to 27-hydroxycholesterol (27-OHC) and regulates cholesterol homeostasis. In Cyp27a1/Apolipoprotein E (ApoE) double knockout (KO) mice fed with Western diet (WD), the atherosclerotic phenotype found in ApoE KO mice was reversed. As protective mechanism, up-regulation of Cyp3a11 and Cyp7a1 was proposed. Cyp27a1 heterozygote/ApoE KO (het) mice, with reduced Cyp27a1 expression and normal levels of Cyp7a1 and Cyp3a11, developed more severe lesions than ApoE KO mice. To analyze the contribution of Cyp3a11 to the protection of atherosclerosis development, Cyp3a11 was induced by Rifampicin (RIF) in ApoE KO and het mice. Males were fed with WD and treated daily with RIF (10 mg/kg ip) or vehicle for 4 weeks. Atherosclerosis was quantified in the aortic valve. Plasma lipids and 27-hydroxycholesterol (27-OHC), expression of cytochromes P450 and genes involved in cholesterol transport and bile acids (BAs) signaling in liver and intestine, and intestinal cholesterol absorption were analyzed. RIF increased expression of hepatic but not intestinal Cyp3a11 4-fold in both genotypes. In ApoE KO mice treated with RIF, we found a 2-fold decrease in plasma cholesterol, and a 2-fold increase in high-density lipoprotein/low-density lipoprotein ratio and CY27A1 activity. Intestinal cholesterol absorption remained unchanged and atherosclerotic lesions decreased approximately 3-fold. In het mice, RIF had no effect on plasma lipids composition, CYP27A1 activity, and atherosclerotic plaque development, despite a reduction in cholesterol absorption. In conclusion, the antiatherogenic effect of Cyp3a11 induction by RIF was also dependent on Cyp27a1 expression.

Introduction

Sterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P450 enzyme which hydroxylates cholesterol at C27 into 27-hydroxycholesterol (27-OHC) and cholestenoic acid [1,2]. CYP27A1 catalyzes the first step of the classic and intermediate step of the alternative pathway of bile acid (BA) biosynthesis [3,4]. It is also involved in cholesterol efflux, the first and rate limiting step of reverse cholesterol transport (RCT). RCT removes cholesterol from extrahepatic tissues to high-density lipoprotein (HDL) and transports it to the liver for elimination via BA. Overexpression of CYP27A1 in CHOP cells or macrophages stimulates efflux [5,6]. In in vivo RCT studies, more [3H]-cholesterol is delivered to the liver and feces following injection of macrophages transfected with a combination of genes containing Cyp27a1 [7]. In vitro, 27-OHC acts as a negative feed-back regulator of the rate limiting enzyme of cholesterol biosynthesis, the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) [8]. Thus, CYP27A1 is a substantial modulator of cholesterol homeostasis, owing its participation in pathways regulating cholesterol elimination and synthesis.

To test the effect of CYP27A1 deficiency on the development of atherosclerosis, we previously crossed Cyp27a1 knockout (KO) mice with Apolipoprotein E (ApoE) KO mice, and fed the offspring with a Western diet (WD) for 3 and 6 months [9]. The atherosclerotic phenotype in ApoE KO mice was reversed in...
Cyp27a1/ApoE double KO (DKO) whereas Cyp27a1 heterozygote/ApoE KO (het) with reduced 27-OHC production developed much more severe lesions than ApoE KO mice. Low plasma cholesterol concentration and enhanced activities of hepatic sterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1), two cytochromes P450 involved in BAs formation, were main contributors to the protective effects in DKO mice. Additionally, increased expression of hepatic cytochrome P450 Cyp3a11 involved in BA detoxification, also contributed to the antiatherosclerotic mechanism.

In humans, CYP3A4, the murine homolog of Cyp3a11, is known to metabolize more than 60 per cent of all therapeutic drugs. It accounts for the oxidative metabolism of compounds used for the treatment of hyperlipidemia (statins), bacterial infections (erythromycin), autoimmune diseases (cyclosporine), diabetes (thiazolidinediones and sulfonylureas), hypertension (calcium channel blockers), and cancer (cytostatics) [10,11]. CYP3A4 also metabolizes numerous endogenous chemicals. Thus, pharmacodynamic approaches modulating CYP3A4 activity offers an attractive option to target therapeutic effects of endo- or xenobiotics metabolized by CYP3A4 in humans. Rifampicin (RIF), often administered as an antibiotic for the treatment of tuberculosis, is a potent CYP3A4 inducer [12]. It exerts its activity via Pregnane X receptor (PXR) activation [13].

Based on our observations in DKO mice [9] and to investigate the relevance of CYP3A11 for cholesterol homeostasis, we analyzed the effect of Cyp3a11 induction by RIF on atherosclerosis in ApoE KO and het mice with reduced Cyp27a1 expression.

**Experimental Chemicals**

RIF (R3501) was purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). [4-14C] Cholesterol and [22,23-3H] β-sitosterol were obtained from American Radiolabeled Chemical, Inc. (St Louis, MO, U.S.A.). All other relevant chemicals are listed under ref. [9].

**Animals**

Animal experimentation was approved by the Ethics Committee for Animal Experiments of the Veterinary Administration of the Canton of Bern, Switzerland, and conformed to the rules of the Swiss Federal Act on Animal Protection Guide for the Care and Use of Laboratory Animals.

ApoE KO mice and their het counterparts (C57BL/6) were bred from het X het mating. Pups were maintained and genotyped as previously published [9]. For the experiments, male ApoE KO and het were weaned at the age of 4 weeks and fed from this age with a WD containing 21% fat and 0.15% cholesterol (Provimi Kliba, Switzerland). At the age of 6 weeks, a first cohort of mice was divided in two groups (n=6) and treated daily with RIF (10 mg/kg ip) or vehicle (0.9% saline) for 4 weeks. At the end of the experiment, mice were starved for 4 h from 8 a.m. to 12 a.m. and killed by pentobarbital injection (300 mg/kg, pentobarbital sodium, USP; Abbott Laboratories, North Chicago, IL, U.S.A.) within the next 3 h. The animals were weighed and blood was collected into a tube containing 20–50 U heparin, centrifuged at 4°C for 15 min at 13000 rpm, and stored at −20°C until use. Organs were removed, one part was washed with PBS and fixed in formalin at 4°C, and the other part was frozen in liquid nitrogen and stored at −70°C. The heart and aorta were fixed in 4% paraformaldehyde for 24 h, transferred to PBS, and stored at 4°C.

A second cohort of ApoE KO and het mice (n=6) was fed at the age of 8 weeks with WD and cholesterol absorption was measured before and 2 weeks following daily injection of RIF (10 mg/kg ip) as described below.

**Characterization of atherosclerotic lesions**

Lesions of the aortic valve were analyzed in paraffin embedded sections of 10 µm at 20 µm intervals for 700 µm and stained with hematoxylin and eosin (H&E) as described previously [9]. Lesion areas were quantified in a blinded manner by image analysis software (ImageJ; NIH, Bethesda, MD, U.S.A.).

**Biochemical analysis of plasma and liver**

Triglycerides (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were quantified in plasma with a kit from Wako Chemicals GmbH (Neuss, Germany). Glucose and alanine aminotransferase (ALT) were measured using an electrochemiluminescence immunoassay from Roche Diagnostics (Zug, Switzerland) respectively Elabscience (Chemie Brunschwig AG, Switzerland). In liver homogenates, TG was measured using a TG quantification kit (BioVision, Mountain View, CA, U.S.A.), cholesterol, and cholesteryl esters (CE) with a CE quantification kit (Calbiochem-Merck Millipore, Zug, Switzerland). 27-OHC was quantified in 100 µl of plasma by gas-chromatography and mass spectrometry (GC-MS). The same
extraction procedure as the one described for HPLC-MS was used [14]. Samples were then derivatized with 100 µl of pyridine and 100 µl of N,O-bis-trimethylsilyl-trifluoroacetamide for 1 h at 60°C, using 5α-cholestan-3β,6α-diol and stigmasterol (100 ng each) as internal standard, and applied on GC-MS [9,15].

**RNA extraction and real-time PCR in liver and jejunum**

RNA extraction and real-time PCR in liver and jejunum homogenates were conducted according to standard procedures as described [9]. Reverse transcription was performed with 2 µg of RNA in a reaction containing 100 U SuperScript Reverse Transcriptase type II (Roche, Basel, Switzerland). Real-time PCR was performed with 100 ng cDNA/reaction. TaqMan assay for Abcg1 (Mm01348250_m1) was ordered from Life Technologies-Applied Biosystems (Switzerland). For Niemann-Pick C1-like 1 (Npc1l1), forward and reverse primers (caacatcttcatctttgttcttgag and gccaatgtgagcctctcg) were from Microsynth (Switzerland) and UPL probes (number 110) from Roche Diagnostics (Germany). The list of primers and probes were described in the previous study [9]. Actb (AM1720) was used as the internal standard. Quantification was performed by the relative quantification method with samples from ApoE KO mice fed WD and treated with vehicle used for calibration.

**Cholesterol absorption**

In a second cohort of mice, gavage was performed with 100 µl of soybean oil containing 0.1 µCi of [4-14C] cholesterol and 0.2 µCi of [22, 23-3H] β-sitosterol. After gavage, each mouse was individually housed in a cage covered with Whatman paper and had free access to food and water. Feces were collected daily for 3 days. Samples were extracted by saponification with 5 M KOH in 50% ethanol and heated at 80°C for 1 h. Lipids were extracted by Folch method. The extracted lipids were transferred into scintillation vials and isotopes were counted in β-counter. The percentage of cholesterol absorption was calculated as follows:

\[
\frac{\text{(14C/3H) dose ratio} - \text{(14C/3H) faeces ratio}}{\text{(14C/3H) dose ratio}} \times 100
\]

**Statistical analysis**

To determine statistically significant differences, ANOVA was used followed by post hoc test (Tukey or Bonferroni) for multiple comparisons.

**Results**

**Effects of RIF on body and organs weight and liver histology**

Administration of RIF to ApoE KO and het mice did not affect body weight, nor liver, spleen, kidney, and brain weights at all time points investigated (Table 1). Treatment with RIF had no effect on general morphology of the liver; no hepatotoxicity was observed under the conditions used (Figure 1), and hepatic transaminase (ALT) remained unchanged (Table 1).

**Effect of RIF on development of atherosclerotic plaque**

A representative picture of atherosclerotic lesions is shown in Figure 2A. Atherosclerotic lesions were significantly reduced upon RIF treatment in ApoE KO mice (0.013 ± 0.003 vs 0.005 ± 0.001 mm², mean ± SEM, P<0.05) but not in het (0.022 ± 0.006 vs 0.015 ± 0.003, mean ± SEM, NS) (Figure 2B). The amount of atherosclerotic plaque tend to be higher in het than in ApoE KO mice, but the difference was significant only in the RIF group (0.015 vs 0.005 mm², P<0.01) (Figure 2A and B). When the relative amount of atherosclerotic plaque was considered, RIF reduced atherosclerosis formation by 66% in ApoE KO mice and by only 38% in het (Figure 2C).

**Effect of RIF on plasma and liver**

Next we analyzed plasma lipid and lipoprotein profile (Table 1). In ApoE KO mice, RIF treatment led to a 2-fold decrease in TC, a 1.5-fold decrease in LDL-C, and 1.5-fold increase in HDL-C; as a result HDL/LDL-C ratio increased 2.5-fold. There was no significant difference in TG levels. CYP27A1 activity, assessed as 27-OHC/TC ratio, was increased by RIF. Plasma glucose was unchanged (Table 1). In the liver, RIF significantly reduced TC but did not lead to any changes in cholesterol ester (CE) or TG in ApoE KO mice.
Table 1 Effect of RIF on organs weights, plasma and hepatic parameters

| Parameter                  | apoE KO   | RIF       | het   | RIF       |
|----------------------------|-----------|-----------|-------|-----------|
| Weight                     | n = 6     | n = 6     | n = 6 | n = 6     | P value   |
| Body (g)                   | 25.6±0.7  | 25.4±0.6  | 24.9±0.4 | 25.2±0.8  | NS        |
| Liver (g)                  | 1.46±0.05 | 1.46±0.04 | 1.38±0.05 | 1.32±0.05 | NS        |
| Kidney (g)                 | 0.15±0.01 | 0.15±0.01 | 0.14±0.01 | 0.15±0.01 | NS        |
| Spleen (g)                 | 0.11±0.01 | 0.12±0.02 | 0.13±0.02 | 0.12±0.03 | NS        |
| Brain (g)                  | 0.46±0.01 | 0.45±0.01 | 0.44±0.01 | 0.43±0.01 | NS        |
| Plasma                     |           |           |       |           |           |
| TC (mmol/l)                | 22.2±5.4  | 9.4±1.9 **| 18.0±2.1 | 17.6±1.0 ***| < 0.0001  |
| LDL-C (mmol/l)             | 17.2±1.7  | 12.2±1.7 ***| 11.7±1.4 ***| 13.3±2.4 | 0.0003    |
| HDL-C (mmol/l)             | 0.57±0.15 | 0.88±0.24 # | 0.57±0.14 | 0.53±0.14 * | 0.0217    |
| HDL/LDL-C                  | 0.03±0.01 | 0.07±0.03 **| 0.05±0.01 | 0.04±0.01 * | 0.0018    |
| TG (mmol/l)                | 1.5±0.6   | 2.1±0.6   | 0.9±0.3 | 1.0±0.3 ** | 0.0002    |
| Glucose (mmol/l)           | 7.5±0.6   | 8.5±0.5   | 6.5±0.5 | 7.5±0.8 | NS        |
| ALT (ng/ml)                | 7.0±2.0   | 7.5±1.5   | 7.4±1.6 | 7.0±1.5 | NS        |
| Markers of CYP27A1 activity|           |           |       |           |           |
| 27-OHC (ng/ml)             | 459±177   | 323±114   | 179±48 ** | 274±54 | 0.0073    |
| CYP27A1 activity (ng/mg)   | 54±17     | 91±41 *   | 30±12 * | 40±8 * | 0.0351    |
| Liver                      |           |           |       |           |           |
| TC (μg/mg)                 | 11.3±1.7  | 8.3±1.0 **| 8.9±1.4 * | 9.1±0.6 | 0.0037    |
| CE (μg/mg)                 | 5.1±1.4   | 5.0±1.0   | 3.6±0.8 | 4.5±0.7 | NS        |
| TG (nmol/mg)               | 32.8±8.7  | 40.0±7.5  | 38.1±11.9 | 33.0±16.4 | NS        |

Results are presented as means ± SD. Significant differences were revealed by two-way ANOVA followed by Bonferroni’s post-test, with # P<0.05, ## P<0.01, ### P<0.001 for the effect of RIF and * P<0.05, ** P<0.01, *** P<0.001 for the effect of the genotype.

Figure 1. Effect of RIF on liver morphology
Representative photomicrographs of H&E liver sections of ApoE KO and het mice fed with WD and treated with 10 mg/kg RIF or vehicle i.p. daily for 4 weeks; scale bars: 20 μm.
Figure 2. Effect of RIF on development of atherosclerosis

Representative photomicrographs of aortic valve in paraffin-embedded sections stained with H&E in ApoE KO and het mice fed with WD and treated with 10 mg/kg RIF or vehicle i.p. daily for 4 weeks; scale bars: 200 μm. (A) Quantification of atherosclerotic lesions in [mm²] (B) and as relative amount of atherosclerotic plaque when compared with vehicle (C). Arrows indicate atherosclerotic plaques. Means with whiskers (minimum and maximum) are presented; *P<0.05, **P<0.01.

In het mice, RIF did not lead to significant changes in plasma lipids (Table 1). When compared with ApoE KO mice, after RIF treatment, het had higher TC levels and lower HDL-C, HDL/LDL-C ratio, TG, and CYP27A1 activity (Table 1). In het mice, RIF had no impact on liver lipids.

Effect of RIF on gene expression in the liver

The effect of RIF on the expression of genes of the cytochrome family, those involved in BAs signaling and cholesterol efflux, was analyzed in the liver (Figure 3). RIF led to a 4-fold increase of the expression of Cyp3a11 in both genotypes. RIF also induced expression of Cyp27a1 and Cyp8b1 in ApoE KO but not in het mice and had no effect on Cyp7a1 expression (Figure 3A). A genotype mediated effect of RIF was seen for the receptors involved in BA signaling (Figure 3B). RIF reduced the expression levels of Nr1h4 (gene for farnesoid receptor (FXR)) and Nr0b2 (gene for small heterodimer partner (SHP)) in het mice, but increased Nr1h4 expression in ApoE KO mice. The analyses of the expression of genes involved in cholesterol efflux revealed that RIF induced the expression of Abca1, Scarb1 (gene for scavenger receptor class B member 1), and Abcg1 in ApoE KO mice (Figure 3C). In het mice, the expression of...
Figure 3. Effect of RIF on gene expression in the liver
The expression of genes encoding for cytochromes P450 (A), BAs signaling (B), and cholesterol efflux (C) was quantified in ApoE KO and het mice fed with WD and treated with 10 mg/kg RIF or vehicle i.p. daily for 4 weeks, using β-actin as housekeeping gene. Results are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, vs ApoE KO, #P < 0.05, ###P < 0.001 vs vehicle.

all the genes involved in cholesterol efflux was higher than in their ApoE KO littermates but did not change upon treatment with RIF (Figure 3C).

Effect of RIF on intestinal cholesterol absorption and gene expression in jejunum
Cholesterol absorption was assessed using the fecal dual-isotope ratio method (Figure 4). The same percentage of cholesterol absorption was observed in vehicle treated ApoE KO and het mice. RIF had no effect on cholesterol absorption in ApoE KO but significantly reduced it in het mice (P < 0.05). To explore the mechanism accounting for the changes in cholesterol absorption, expression of cytochromes P450 (Figure 5A), genes involved in BAs signaling (Figure 5B), and cholesterol transport (Figure 5C) were analyzed in the intestine. RIF had no effect on Cyp3a11 expression in the jejunum in ApoE KO or het mice but down-regulated Cyp27a1 mRNA levels in het mice (Figure 5A). RIF reduced expression of Nr1h4, Nr0b2, and fgf15 (fibroblast growth factor 15) to the same magnitude in both genotypes and had no effect on expression of Nr1i2 (gene for PXR) (Figure 5B). In ApoE KO mice, RIF had no significant effect on the expression level of genes involved in cholesterol transport (Figure 5C). In het, RIF slightly induced Nr1h3 (gene for LXR α) expression and slightly reduced Abca1 expression.
Figure 4. Effect of RIF on cholesterol absorption
Cholesterol absorption was measured in ApoE KO and het mice fed with WD and following treatment with 10 mg/kg RIF or vehicle i.p. daily, using dual absorption method. Results are presented as mean ± SEM; *P<0.05, **P<0.01.

Figure 5. Effect of RIF on cholesterol homeostasis in the jejunum
The expression of genes encoding for cytochromes P450 (A), BAs signaling (B), and cholesterol efflux (C) was quantified in ApoE KO and het mice fed with WD and treated with 10 mg/kg RIF or vehicle i.p. daily for 4 weeks using β-actin as housekeeping gene. Results are presented as mean ± SEM; *P<0.05, vs ApoE KO, #P<0.05, ##P<0.01, ###P<0.001 vs vehicle.

Discussion
In our previous study [9], DKO mice fed with WD developed 10-fold less atherosclerosis than their ApoE KO littermates. In contrary, het mice with reduced Cyp27a1 expression had much more atherosclerosis than ApoE KO mice. Among the proposed protective mechanisms in DKO mice, increased cholesterol metabolism via overexpression of hepatic Cyp3a11 was proposed. CYP27A1 was also atheroprotective, since het mice with reduced plasma 27-OHC concentration and normal levels of Cyp3a11 expression developed more atherosclerotic lesions than ApoE KO mice. To investigate the influence of cholesterol metabolism by CYP3A11 and CYP27A1 on development of atherosclerosis, RIF, a known inducer of Cyp3a11, was administrated to ApoE KO and het mice.

The key findings of our study can be summarized as follows (Figure 6). First, RIF protected ApoE KO mice fed a
proatherogenic diet from atherosclerosis development. Second, RIF induced Cyp3a11 hepatic expression to the same levels in ApoE KO and het mice. Third, RIF had a specific effect on CYP27A1 in ApoE KO mice, as demonstrated by (i) increased hepatic expression of Cyp27a1 and (ii) elevated apparent CYP27A1 activity, measured as 27-OHC/TC ratio in plasma. Finally, RIF did not induce intestinal Cyp3a11. In ApoE KO mice, RIF had no influence on cholesterol absorption. In contrary in het mice, RIF slightly reduced the expression of Cyp27a1 and cholesterol absorption. These observations point to the importance of an adequate expression of Cyp27a1 to protect against atherosclerosis development in vivo in mice.

The antiatherosclerotic effect of RIF in ApoE KO mice was likely due to its effect on plasma lipids and lipoproteins. Favorable changes in lipid profile consisting in a decrease in plasma TC and LDL-C combined with an increased in plasma level of HDL-C and increased CYP27A1 activity, is a likely reason for which ApoE KO mice treated with RIF developed less atherosclerosis than their vehicle-treated littermates. RIF also significantly increased the expression of Cyp27a1, Abca1, Scarb1, and Abcg1 in the liver, all four genes involved in cholesterol efflux. The effect of RIF on development of atherosclerosis was less pronounced in het mice with reduced Cyp27a1 expression. The plasma proatherogenic properties, such as high TC, high LDL-C, low HDL-C, and low apparent CYP27A1 activity, remained unchanged upon treatment with RIF in het mice. Furthermore, the expression of hepatic genes involved in cholesterol efflux was not induced by RIF in het mice. Cyp27a1 expression was slightly decreased in the intestine, but the decrease

Figure 6. Cyp27a1 expression and the antiatherosclerotic effect of RIF in mice
The effect of Cyp3a11 induction by RIF on atherosclerosis development was analyzed in ApoE KO mice with normal Cyp27a1 expression and 27-OHC plasma concentration, and in het mice with reduced Cyp27a1 expression and 27-OHC plasma concentration. RIF induced hepatic Cyp3a11 mRNA 3-fold and had no effect on intestinal Cyp3a11 expression in both genotypes. In ApoE KO mice, RIF increased hepatic Cyp27a1 mRNA, leading to an increase in CYP27A1 activity measured by the 27-OHC/Cholesterol ratio in plasma. RIF also decreased TC and LDL-C and increased HDL-C. In het mice, RIF reduced Cyp27a1 mRNA levels in the intestine and had no effect on plasma lipids, despite reduced cholesterol absorption. The different effect of RIF on atherosclerosis development in ApoE KO and het mice underline the importance of Cyp27a1 expression in the protection of atherosclerosis.
in intestinal cholesterol absorption was not sufficient to decrease atherosclerotic plaque to the level of this observed in ApoE KO mice. Based on these findings, we conclude that, first, the differences in the effect of RIF on development of atherosclerosis was mainly due to the level of Cyp27a1 expression in the liver and, second, that RIF treatment was less effective when Cyp27a1 expression was reduced.

Our findings on lipoprotein profile changes in plasma of ApoE KO mice are consistent with a previous study from Bachmann et al. [16]. In this work, induction of Cyp3a11 with imidazole in rats and with RIF in mice led to increased levels of plasma HDL-C and apoA-1. The effect was mediated by PXR, since similar treatment in wild-type and Pxr KO mice had a different effect on plasma HDL-C and apoA-1 [16].

Although the effect of RIF on human PXR is well established, we have shown in the present study that RIF can also increase Cyp3a11 expression in non-humanized mice. This result contrasts with several reports that failed to show any induction of Cyp3a11 in the liver. This discrepancy can be explained as follows. First, much shorter treatments (4–5 days) were used in these studies [17–19]. Second, the mode of administration differed. Since we applied RIF i.p. and not by gavage, the effect of gastric acid on the drug was avoided. Finally, the genetic background of the animals was different; Hosagrahara et al. [18] used BALB/c mice, while our studies were performed in C57BL/6 mice.

Enhanced expression of Cyp27a1 in addition to the induction of Cyp3a11 expression is an interesting finding, given the role of CYP27A1 in cholesterol efflux [5]. In het mice with reduced CYP27A1 activity, the effect of RIF on hepatic Cyp27a1 was less pronounced, and plasma level of TC was higher. Our results are in line with those of Sheng et al. [20] who described a direct effect RIF on hepatic Cyp27a1 mRNA expression in ICR mice. A 3 weeks treatment with 100 mg/kg/day led to an increase in serum 25-hydroxy vitamin D3, a metabolite obtained from the conversion of vitamin D3 by CYP27A1 in the liver.

Whether our findings can be extrapolated to humans remains to be seen, given the profound dissimilarities between bile acids metabolism between humans and mice. Indeed, the phenotype of Cyp27a1 KO mice was mainly expressed in the liver, with a severe microvesicular steatosis, whereas CYP27A1 deficiency in humans with Cerebrotendinous Xanthomatosis (CTX), clinical manifestations include bilateral juvenile cataracts and accumulation of cholesterol in different tissues [21]. For many years, RIF was used as an antibiotic for the treatment of tuberculosis and it is still used in clinical practice [12]. CTX, patients, induction of CYP3A4 activity by RIF cannot replace the effective therapy with chenodeoxycholic acid (CDCA). Whereas CDCA treatment leads to the inhibition of cholesterol and 7α-hydroxycholesterol synthesis and a reduction in plasma cholestanoles concentration, upon RIF treatment, cholesterol levels remain unchanged, despite a 60% increase in CYP3A4 activity [22]. The difference of effect between the two substances could be explained, at least in part, by the missing effect of RIF on CYP27A1, in the absence of CYP27A1 expression in CTX patients. In healthy volunteers, daily treatment with 600 mg RIF has no effect on normal plasma cholesterol at first, but after a week, it increases cholesterol steadily [23]. The same dose of RIF induces hypercholesterolemia in patients with acute brucellosis treated with a bitherapy consisting of RIF (600 mg/day) and Doxycycline (100 mg/day) [24]. Thus, in humans, treatment with RIF may not be as beneficial as in mouse models. However, in these studies, 27-OHC (which we use as marker for CYP27A1 activity to calculate the 27-OHC/cholesterol ratio) was not measured, making mechanistic conclusions about the effect of RIF on lipoprotein profile in humans difficult.

There are some limitations in the present study. The effect of RIF on atherosclerosis development was performed on young mice, at an early stage of atherosclerosis development. Whereas this remains true in elderly mice with more severe atherosclerotic lesions, remains to be proven. The length of the treatment was also relatively short. In order to avoid hepatotoxicity, RIF was administered for 4 weeks only. This is a relative short time frame for atherosclerosis development, even in the ApoE KO model with WD. Although we observed a marked induction of Cyp3a11 in both genotypes, the level of increase was not related to PXR activation, since our mice were not crossed with PXR humanized mice. Finally, the mechanisms behind the observed effects remains uncertain. A putative mechanism, based on available data, is depicted in Figure 6. Further studies, such as knocking down Cyp3a11 in ApoE KO and het mice would largely contribute to the clarification of the role of Cyp27a1 and Cyp3a11 in atherosclerosis development.

In conclusion, despite a similar induction level of Cyp3a11 expression in the liver, RIF had a different effect on plasma lipids and atherosclerosis development in young ApoE KO and het mice, revealing the importance of Cyp27a1 gene dosage in vivo in mice.

Acknowledgments
We thank Ms. Beatrice Rohrbach for technical help and Dr Anne-Christine Uldry for assistance in statistics.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.
Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contribution
Participated in research design: Geneviève Escher and Line Zurkinden; Conducted experiments: Line Zurkinden; Performed data analysis: Line Zurkinden, Dmitri Sviridov, and Geneviève Escher; Wrote or contributed to the writing of the manuscript: Geneviève Escher, Bruno Vogt, and Dmitri Sviridov.

Abbreviations
27-OHC, 27-hydroxycholesterol; ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; ACTB, Actin; ApoA-1, apolipoprotein A-I; ApoE, apolipoprotein E; BA, bile acid; CDCA, chenodeoxycholic acid; CE, cholesteryl ester; CTX, cerebrotendinous xanthomatosis; CYP3A11, cytochrome P450 3A11; CYP27A1, sterol 27-hydroxylase; CYP7A1, cytochrome P450 enzyme 7α-hydroxylase; CYP8B1, sterol 12-alpha-hydroxylase; DKO, Cyp27a1 KO/ApoE KO mice; FG515, fibroblasts growth factor 15; FXR, farnesoid X receptor; GC-MS, gas chromatography - mass spectrometry; HDL-C, high-density lipoprotein cholesterol; H&E, hematoxylin and eosin; Het, Cyp27a1 heterozygote/ApoE KO mice; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; KO, knockout; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LXR, liver X receptor; NPC1L1, Niemann-Pick C1-like protein 1; PXR, pregnane X receptor; RCT, reverse cholesterol transport; RIF, Rifampicin; SHP, small heterodimer partner; TG, triglyceride; WD, Western diet.

References
1 Norlin, M. et al. (2003) On the substrate specificity of human CYP27A1: implications for bile acid and cholestanol formation. J. Lipid Res. 44, 1515–1522, https://doi.org/10.1194/jlr.M300047-JLR200
2 Cali, J.J. et al. (1991) Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. J. Biol. Chem. 266, 7779–7783
3 Björkhem, I. (1992) Mechanism of degradation of the steroid side chain in the formation of bile acids. J. Lipid Res. 33, 455–471
4 Russell, D.W. and Setchell, K.D. (1992) Bile acid biosynthesis. FASEB J. 6, 337–348, https://doi.org/10.1002/fj.260060715
5 Zurkinden, L. et al. (2014) Effect of Cyp27a1 gene dosage on atherosclerosis development in ApoE-knockout mice. J. Lipid Res. 55, 2531–2542, https://doi.org/10.1194/jlr.M300047-JLR200
6 Guengerich, F.P. et al. (2000) What makes P450s work? Searches for answers with known and new P450s. Am. J. Physiol. 278, G266–G278, https://doi.org/10.1152/ajpgi.2000.278.3.G266
7 Escher, G. et al. (2003) Expression of sterol 27-hydroxylase (CYP27A1) enhances cholesterol efflux. J. Biol. Chem. 278, 11015–11019, https://doi.org/10.1074/jbc.M212780200
8 Isabelle Vögeli, H.H.J., Dick, B., Erickson, S.K., Escher, R., Funder, J.W., Frey, F.J. et al. (2013) Evidence for a role of sterol-27 hydroxylase in glucocorticoid metabolism in vivo. J. Endocrinol., in press
9 Hall, E. et al. (2001) Overexpression of CYP27 in hepatic and extrahepatic cells: role in the regulation of cholesterol homeostasis. Am. J. Physiol. Gastrointest. Liver Physiol. 281, G293–G301
10 Raybon, J.J. et al. (2011) Pharmacokinetic-pharmacodynamic modeling of rifampicin-mediated Cyp3a11 induction in steroid and xenobiotic X receptor humanized mice. J. Pharmacol. Exp. Ther. 337, 37, https://doi.org/10.1124/jpet.111.176677
11 Hasegawa, M. et al. (2012) Investigation of drug-drug interactions caused by human pregnane X receptor-mediated induction of CYP3A4 and CYP2C subfamilies in chimeric mice with a humanized liver. Drug Metab. Dispos. 40, 474–480, https://doi.org/10.1124/dmd.111.042754
12 Medellin-Garibay, S.E. et al. (2014) Pharmacokinetics of rifampicin in Mexican patients with tuberculosis and healthy volunteers. J. Pharm. Pharmacol. 66, 1421–1428, https://doi.org/10.1111/jphp.12275
13 Norlin, M. et al. (2003) On the substrate specificity of human CYP27A1: implications for bile acid and cholestanol formation. J. Lipid Res. 44, 1515–1522, https://doi.org/10.1194/jlr.M300047-JLR200
14 Bachmann, K. et al. (2004) PXR and the regulation of apoA1 and HDL-cholesterol in rodents. Pharmacol. Res. 50, 237–246, https://doi.org/10.1016/j.phrs.2004.03.005
15 Sheng, L. et al. (2015) Effects of repeated administration of rifampicin and isoniazid on vitamin D metabolism in mice. Steroids 104, 203–207, https://doi.org/10.1016/j.steroids.2015.10.004
21 Bjorkhem, I. (2013) Cerebrotendinous xanthomatosis. Curr. Opin. Lipidol. **24**, 283–287, https://doi.org/10.1097/MOL.0b013e328362df13
22 Szalat, A. et al. (2007) Rifampicin-induced CYP3A4 activation in CTX patients cannot replace chenodeoxycholic acid treatment. Biochim. Biophys. Acta **1771**, 839–844, https://doi.org/10.1016/j.bbalip.2007.04.012
23 Kasichayanula, S. et al. (2014) Validation of 4beta-hydroxycholesterol and evaluation of other endogenous biomarkers for the assessment of CYP3A activity in healthy subjects. Br. J. Clin. Pharmacol. **78**, 1122–1134, https://doi.org/10.1111/bcp.12425
24 Apostolou, F. et al. (2009) Persistence of an atherogenic lipid profile after treatment of acute infection with Brucella. J. Lipid Res. **50**, 2532–2539, https://doi.org/10.1194/jlr.P900063-JLR200