Statins improve NASH via inhibition of RhoA and Ras

© Robert Schierwagen,1 Lara Maybüchen,1 Kanishka Hittatiya,2 Sabine Klein,1 Frank E. Uschner,1 Tarcio T. Braga,3 Bernardo S. Franklyn,3 Georg Nickenig,4 Christian P. Strassburg,1 Jogchum Plat,5 Tilman Sauerbruch,1 Eicke Latz,1 Dieter Lütjohann,6 Sebastian Zimmer,3* and Jonel Trebicka1,7*

1Department of Internal Medicine I, University of Bonn, Bonn, Germany; 2Institute of Pathology, University of Bonn, Bonn, Germany; 3Institute of Innate Immunity, University of Bonn, Bonn, Germany; 4Department of Internal Medicine II, University of Bonn, Bonn, Germany; 5Department of Human Biology, University of Maastricht, Maastricht, The Netherlands; 6Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany; and 7Faculty of Health Sciences, Odense University Hospital, Odense, Denmark

Submitted 11 February 2016; accepted in final form 18 August 2016

Statins improve NASH via inhibition of RhoA and Ras. Am J Physiol Gastrointest Liver Physiol 311: G724–G733, 2016. First published September 15, 2016; doi:10.1152/ajpgi.00063.2016.—Nonalcoholic steatohepatitis (NASH), especially as part of the metabolic syndrome (MS), is an increasing burden in Western countries. Statins are already used in MS and seem to be beneficial in liver diseases. The aim of this study was to investigate the molecular mechanisms underlying pleiotropic effects on small GTPases of statins in NASH. NASH within MS was induced in 12-wk-old apoE−/− mice after 7 wk of Western diet (NASH mice). Small GTPases were inhibited by activated simvastatin (SMV), NSC23766 (NSC), or Clostridium sordellii lethal toxin (LT) by using subcutaneous osmotic minipumps. Hepatic steatosis, inflammation, and fibrosis were assessed by histology, Western blot, and RT-PCR measurements of cholesterol and hydroxyproline content. SMV treatment significantly decreased hepatic inflammation and fibrosis, but had no significant effect on steatosis and hepatic cholesterol content in NASH. SMV blunted fibrosis due to inhibition of both RhoA/Rho kinase and Ras/ERK pathways. Interestingly, inhibition of RAC1 and Ras (by LT) failed to decrease fibrosis to the same extent. Inhibition of RAC1 (by NSC) showed no significant effect at all. Inhibition of RhoA and Ras downstream signaling by statins is responsible for the beneficial hepatic effects in NASH.

NASH; liver fibrosis; statins; apoE; Western diet; GTPase

NEW & NOTEWORTHY

Statins are established cholesterol-lowering drugs and have additional pleiotropic properties. This work demonstrates that treatment with activated simvastatin decreased hepatic inflammation and fibrosis in mice with nonalcoholic fatty liver disease, without changing hepatic steatosis. These effects are mediated through the inhibition of RhoA and Ras signaling.

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD), including nonalcoholic steatohepatitis (NASH), within metabolic syndrome (MS) is an increasing health burden (21, 22, 34, 35, 40). Furthermore, patients with MS and NASH are prone to develop cardiovascular diseases (CVD) (4, 39). Today, statins are standard of care in the treatment of MS and CVD (5). Statins are primarily known as cholesterol-lowering drugs by inhibiting HMG-CoA reductase, a central component of cholesterol biosynthesis. But the effects of statins exceed cholesterol lowering, and additional effects are summarized as pleiotropic effects. Inhibition of HMG-CoA reductase entails decreased isoprenoid levels downstream of the pathway. Prenylation, an essential reaction for enzyme function, enables cell membrane anchoring of proteins, e.g., small GTPases and their interaction with downstream effectors (5, 18). Thereby statins modify intracellular signaling of different receptors.

Statins have been shown to blunt liver fibrosis development and decrease portal pressure in animal models (47). Previously, we could show that atorvastatin treatment lowers portal pressure in cirrhotic rats mediated by inhibition of the RhoA/Rho kinase pathway (42). Furthermore, atorvastatin decreases hepatic fibrosis by inhibition of hepatic stellate cell (HSC) activity and is correlated with altered collagen turnover (17, 36, 43). Additional to the demonstrated effects via RhoA/Rho kinase, statins also induce beneficial effects via Krüppel-like factor 2 (KLF2) (23, 47). These studies could be confirmed in human cirrhosis (1, 51) and in further animal models of liver cirrhosis (2, 11, 23, 24). Statins have been shown to be protective in CVD, especially atherosclerosis, mainly through their pleiotropic effects on small GTPases RhoA, Ras, and RAC1 (18, 30, 31, 38).

Interestingly, recent studies could prove that statin treatment ameliorates human NASH (6, 9). Since NASH patients show increased risk for development of CVD (4), statins may not only improve NASH but also prevent the development of concomitant diseases like CVD. However, the underlying molecular mechanisms are not fully understood.

The aim of this study was to dissect the molecular mechanism of statins in NASH by using Western diet-fed apoE−/− mice (further referred to as NASH mice) exhibiting major characteristics of human NASH.

MATERIALS AND METHODS

Animals. Twelve-week-old apoE-knockout mice (apoE−/−, C57BL/6J background; Charles River, Wilmington, DE) were used. The mice were kept at 22°C with a 12-h:12-h day-night cycle and received water and chow ad libitum. Mice were fed a high-fat, cholesterol-rich diet [Western diet (WD)] containing 21% fat (with coconut oil), 19.5% casein, and 1.25% cholesterol (Sniff, Soest, Germany) for 7 wk as described previously (further referred to as NASH mice) (12, 37, 41). To characterize the extent of liver damage at the point of treatment initiation, mice were either fed normal diet (ND) or WD for 1 wk. All experiments were performed in accordance with the German Animal-Protection Law and the guidelines of the animal care unit at our university (Haus für experimentelle Therapie, Bonn, Germany) and approved by the

* S. Zimmer and J. Trebicka share last authorship.

Address for reprint requests and other correspondence: J. Trebicka, Dept. of Internal Medicine I, Univ. of Bonn, Sigmund-Freud Str. 25, D-53127 Bonn, Germany. (e-mail: jonel.trebicka@ukb.uni-bonn.de).

**Osmotic minipumps.** To elucidate the role of different small GTPases on fibrogenesis in NASH, subcutaneous osmotic minipumps were implanted under anesthesia 1 wk after onset of the WD. Over a period of 6 wk, these pumps administered either simvastatin (SMV) (RhoA/Ras/RAC1 inhibitor, 20 mg·kg⁻¹·day⁻¹), Clostridium sordellii lethal toxin (LT) (Ras/RAC1 inhibitor, 0.1 mg·kg⁻¹·day⁻¹), or solvent (sodium chloride 0.9%) (Fig. 1A). SMV is an inactive lactone prodrug that is converted in vivo to the active open-ring acid form. Thus an alkaline hydrolysis procedure was performed to reach the active form before administration to mice (19). LT belongs to the family of clostridial cytotoxins and inactivates RhoA and Ras GTTPases by monoglycosylation at a site critical for GTP binding (15).

**Tissue collection and determination of hepatic cholesterol lipoprotein fractions.** Mice were anesthetized and laparotomy was performed for tissue collection after 6 wk of treatment. The liver was cut into fragments. As described previously, liver samples were either snap frozen in liquid nitrogen and stored at −80°C or fixed in formaldehyde (4%) (42, 43). Serum lipoprotein profiles were determined by fast protein liquid chromatography as described earlier (7).

**Hepatic hydroxyproline content.** Hepatic hydroxyproline was photometrically measured in liver hydrolysates as previously described (42, 43). Briefly, analog segments (200 mg) of snap-frozen livers were processed as previously described with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and nitrocellulose membranes (42, 44). Ponceau S staining was performed to assure equal protein loading, while GAPDH served as endogenous control. The membranes were blocked and incubated with the primary antibodies listed in Table 1. Thereafter, the membranes were incubated with the corresponding secondary peroxidase-coupled antibody (Calbiochem, San Diego, CA). Blots were developed with enhanced chemiluminescence (ECL) (Amersham, UK). Intensities of the digitally detected bands were evaluated with Chemi-Smart (PeqLab Biotechnologies, Erlangen, Germany). Rho kinase activity was assessed as phosphorylation of the endogenous Rho kinase substrate moesin at Thr-558 (F4/80; Mm00802529_m1), Il1b (IL1β; Mm01336189_m1), Ccl2 (MCP1; Mm00441242_m1), Tgfβ1 (TGFβ; Mm03024053_m1), and Tnfa (TNFα; Mm00443258_m1). Results were normalized to 18S rRNA of the respective sample, were expressed as 2⁻ΔΔCt, and demonstrate the x-fold shift of gene transcription compared with solvent injected apoE⁻⁻ control NASH mice.

**Western blot analysis.** Samples of snap-frozen livers were processed as previously described with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and nitrocellulose membranes (42, 44). Ponceau S staining was performed to assure equal protein loading, while GAPDH served as endogenous control. The membranes were blocked and incubated with the primary antibodies listed in Table 1. Thereafter, the membranes were incubated with the corresponding secondary peroxidase-coupled antibody (Calbiochem, San Diego, CA). Blots were developed with enhanced chemiluminescence (ECL) (Amersham, UK). Intensities of the digitally detected bands were evaluated with Chemi-Smart (PeqLab Biotechnologies, Erlangen, Germany). Rho kinase activity was assessed as phosphorylation of the endogenous Rho kinase substrate moesin at Thr-558.

![Liver damage at treatment initiation](image)

Fig. 1. Liver damage at treatment initiation. A: representative sections and quantification of Oil Red O staining show increased accumulation of fat in mice fed Western diet (WD) compared with mice fed normal diet (ND). B: transcription levels of proinflammatory markers were significantly increased in mice fed WD. C: profibrotic markers and markers of hepatic stellate cell activation were significantly increased in mice fed WD. D: apoE⁻⁻ mice fed WD showed increased hepatic collagen deposition. Data are presented as means ± SD. The scale bar is 50 μm for Oil Red O staining. P < 0.05 was considered significant.

**Table 1. Antibodies for Western blot analysis**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Product No.</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>ab5694</td>
<td>Abcam</td>
</tr>
<tr>
<td>β-Actin</td>
<td>926–42212</td>
<td>LI-COR Biosciences</td>
</tr>
<tr>
<td>Caspase 1</td>
<td>AG-20B-0042-C100</td>
<td>AdipoGen Life Sciences</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>sc-6487</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>sc-93</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>GAPDH</td>
<td>sc-25778</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Moesin</td>
<td>3150</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>4370P</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>pMoesin</td>
<td>sc-12895-R</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Ras</td>
<td>3965</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>RhoA</td>
<td>sc-133</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Rho kinase</td>
<td>sc-418</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Rho kinase</td>
<td>sc-5561</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>
Activity of ERK was assessed by its phosphorylation at Thr202/Tyr204.

**Histological staining.** For detection of fat accumulation in the liver, 6-μm-thick sections of snap-frozen liver samples were prepared with a cryostat. Sections were dehydrated overnight, fixed in 10% formalin, washed with 60% Isopropanol alcohol, and stained with Oil Red O (3%) followed by counterstaining with hematoxylin as described previously (28, 37, 46, 48). To detect collagen fibers, paraffin-embedded sections (2–3 μm) were treated with 0.1% Sirius red F3B in saturated picric acid (Chroma, Münster, Germany) as described previously (42, 43). Immunohistochemical staining for αSMA, Ki67, and F4/80 were performed in paraffin-embedded sections (2–3 μm). The sections were incubated with a mouse anti-SMA antibody (Actin clone 1A4; Dako, Hamburg, Germany), rabbit anti-Ki67 (clone SP6; Abcam, Cambridge, UK), or rat anti-F4/80 (clone BM8; BMA Dianova, Hamburg, Germany). Thereafter, a biotinylated goat antimouse (Dako), goat antirabbit (Dako), or rabbit antirat (Biozol, Eching, Germany) secondary antibodies were used, respectively. Finally, sections were counterstained with hematoxylin. Histological staining was digitalized with Pannoramic MIDI (3DHistech, Budapest, Hungary). For computational analysis (Histoquant, 3DHistech), large bile ducts and vessels were excluded, following the principles of computational analysis as described previously (13, 46).

**Statistical analysis.** Data are presented as means ± SD. Statistical analysis of two groups was performed with Mann-Whitney U-test and Kruskal-Wallis test with Dunn’s post hoc test for comparisons of more than two groups using GraphPad Prism (La Jolla, CA) (37). Group size was at least n = 5 for each group. P values < 0.05 were considered statistically significant.

**RESULTS**

**Liver damage at treatment initiation.** At the start of the treatment with GTPase inhibitors or SMV after 1 wk of WD, mice already developed significant steatosis (Fig. 1A). Transcription of proinflammatory genes IL1β, Tnfα, Ccl2, and F4/80 were also significantly increased after 1 wk of WD compared with littersmates fed ND (Fig. 1B). Furthermore, gene expression of αSMA, a surrogate marker for HSC activation, was increased after 1 wk of WD, leading to an increase of profibrotic markers Tgfβ and Col1α1 (Fig. 1C). As a consequence, hepatic collagen accumulation was also increased (Fig. 1D).

**Effect of GTPase inhibitors and SMV on hepatic proliferation and steatosis.** Administration of GTPase inhibitors (NSC and LT) or SMV showed no impact on the proliferation of inflammatory cells compared with untreated NASH mice, as shown by representative staining of Ki67 and their quantification (Fig. 2, B and C).

After 7 wk of WD the serum lipoprotein profiles of apoE−/− NASH mice showed a profound shift toward increased VLDL levels as reported previously (37). Treatment with GTPase inhibitors improved the lipoprotein profile of these mice (Fig. 2D). Mice treated with the RAC1 inhibitor NSC showed highest drop in VLDL levels and further increased HDL levels, whereas LT (RAC1/Ras inhibitor) and SMV (RAC1/Ras/RhoA inhibitor) treatment showed a moderate decrease in VLDL and no overall impact on HDL levels (Fig. 2D). The quantification of Oil Red O stainings revealed no significant changes in hepatic fat accumulation after treatment with any inhibitor compared with untreated NASH mice (Fig. 2, E and F).

**Effect of GTPase inhibitors and SMV on hepatic inflammation.** Hepatic inflammation was assessed by immunohistochemistry of the murine macrophage marker F4/80 and by gene expression levels of inflammatory markers. Treatment with NSC (RAC1 inhibitor), LT (RAC1/Ras inhibitor), or SMV (RAC1/Ras/RhoA inhibitor) after 7 wk of WD led to significantly decreased hepatic inflammation compared with untreated controls, with a slightly more pronounced decrease after treatment with SMV (Fig. 3, A and B). Supporting the findings observed in F4/80 immunohistochemistry, mRNA levels of F4/80 were significantly decreased in mice treated with SMV (Fig. 3C).

Further proinflammatory genes experienced a significant drop only after treatment with SMV compared with untreated NASH mice (Fig. 3D). Treatment with NSC or LT showed only a trend toward a decreased transcription of Il1b (Fig. 3D). Protein levels of caspase-1 were decreased in mice treated with NSC and LT (Fig. 3E).

**Effect of GTPase inhibitors and SMV on hepatic fibrosis.** After 6 wk of treatment, SMV (RAC1/Ras/RhoA inhibitor) led to decreased levels of Sirius red positive staining compared with untreated littersmates (Fig. 4, A and B). Treatment with neither NSC (RAC1 inhibitor) nor LT (RAC1/Ras inhibitor) decreased hepatic collagen accumulation (Fig. 4, A and B). Furthermore, content of hepatic hydroxyproline, as a major component of collagen, was decreased significantly in mice by either SMV or LT treatment (Fig. 4C). The treatment with NSC showed only a slight but not significant tendency toward a decrease of hepatic hydroxyproline content compared with control NASH mice (Fig. 4C).

In addition to the findings observed on protein level, profibrotic genes were also downregulated significantly only in SMV-treated mice compared with untreated NASH mice (Fig. 4D). Neither treatment with NSC nor with LT decreased the investigated profibrotic genes significantly, but they partly showed a slight trend toward a decrease (Fig. 4D).

**Effect of GTPase inhibitors and SMV on HSC activation.** Major producers of collagen accumulation and deposition in liver fibrosis are activated HSCs (25). HSC activation was investigated by the surrogate marker αSMA. After 6 wk of treatment with NSC (RAC1 inhibitor), LT (RAC1/Ras inhibitor), or SMV (RAC1/Ras/RhoA inhibitor), αSMA-positive staining was decreased. However, only SMV showed a significant decrease in αSMA-positive staining compared with the untreated NASH mice (Fig. 5, A and B). Findings on the protein level were confirmed by transcriptional levels of αSMA, which showed a significant decrease in NASH mice treated with SMV compared with untreated littersmates (Fig. 5C).

Besides αSMA immunohistochemistry and measurements of gene expression, confirmative Western blot analysis demonstrates a significant decrease of αSMA in mice treated with SMV, whereas there was only a tendency toward decreased αSMA in mice treated with NSC or LT (Fig. 5D). Furthermore, cathepsin D, as a marker for HSC proliferation and their fibrogenic potential, was decreased by SMV treatment compared with the respective control NASH mice (Fig. 5D). Again, treatment with either NSC or LT revealed only a trend toward a decrease of cathepsin D in Western blot analysis (Fig. 5D).

**Inhibition of Ras/ERK and RhoA/Rho kinase signaling is responsible for the SMV effect on hepatic fibrosis.** While NSC selectively inhibits RAC1, LT inhibits RAC1 and Ras, and SMV inhibits RAC1, Ras, and RhoA. Treatment with SMV showed the most beneficial effect on NASH. SMV elicited the
Fig. 2. Effect of GTPase inhibitors and SMV on hepatic proliferation and steatosis. 

**A**: schematic overview of GTPases downstream of G protein-coupled receptors (GPCRs) and targets of simvastatin (SMV), NSC23766 (NSC), and lethal toxin from *Clostridium sordellii* (LT) for GTPase inhibition. **B** and **C**: quantification and representative sections of Ki67 immunohistochemistry as a marker of proliferation showed no effect by inhibitor treatment. **D**: serum lipoprotein profiles showed improvement mainly in the VLDL fraction after treatment with any of the inhibitors compared with untreated NASH mice (+ solvent). NSC23766 (RAC1 inhibitor) showed further beneficial effects on the HDL fractions (inset). **E** and **F**: quantification and representative sections of Oil Red O staining, as a marker of fat accumulation in the liver, showed no significant effect of GTPase inhibition. Data are presented as means ± SD. The scale bar is 50 μm for Ki67 immunohistochemistry and 100 μm for Oil Red O staining. *P* < 0.05 was considered significant.
Effect of GTPase inhibitors and simvastatin (SMV) on hepatic inflammation. A and B: representative sections and quantification of F4/80 histological staining as macrophage marker in the liver. Most pronounced decrease of F4/80 staining after SMV (inhibitor of RAC1, Ras, and RhoA) administration compared with control NASH mice (+ solvent). C: transcription levels of Adgre1 (F4/80) showed significant decrease only after treatment with SMV. D: transcription levels of proinflammatory markers were significantly lower after treatment with SMV compared with control NASH mice, while NSC23766 (RAC1 inhibitor) and lethal toxin from Clostridium sordellii (LT; RAC1 and Ras inhibitor) treatment showed only a trend for Il1b. E: protein expression of caspase 1 was reduced after treatment with NSC or LT with no significant change after treatment with SMV. Data are presented as means ± SD. The scale bar is 100 μm for F4/80 immunohistochemistry. *p < 0.05 was considered significant.
inhibition of both Ras/ERK and RhoA/Rho kinase pathways. RhoA, Rho kinase, and pMoesin, as a marker for Rho kinase activity, were downregulated only in SMV-treated mice (Fig. 6, A and C). LT and NSC showed no effect on RhoA signaling. Similarly, the components of Ras/ERK pathway Ras, RAF1, and activated ERK1/2 (pERK1/2) were downregulated in SMV-treated mice compared with LT, NSC, and control NASH mice with solvent as shown by Western blot analysis (Fig. 6, B and C). A trend toward downregulation of Ras signaling was seen in LT-treated mice, possibly explaining the moderate beneficial effects observed regarding fibrosis (Fig. 6, B and C).

**DISCUSSION**

This study elucidates the molecular mechanisms underlying the beneficial effects of SMV in NASH in a murine model with MS. SMV acts in an antifibrotic and anti-inflammatory manner via the inhibition of the Ras/ERK and RhoA/Rho kinase pathways.

NAFLD is an increasing health burden, since it has become a chronic liver disease in Western countries (3, 21, 40). Statins are a cornerstone in the treatment of MS (5), and they seem to be safe even at high dosages (20). Moreover, patients with MS and NAFLD have a high risk for development of CVDs, especially atherosclerosis (4, 39). Statins have been shown to act beneficially in CVD (18) and may improve atherosclerosis not only by lowering cholesterol levels but also by their pleiotropic effects on inflammation (29). Furthermore, the benefits of statins in NAFLD overrule the potential risks of hepatotoxic effects (32).

Although MS and CVD are commonly associated with NAFLD, statin application in treatment of fatty liver is de-
bated. Previously, only a few studies with a small number of patients have analyzed the effect of statins in human NAFLD and NASH (8, 14, 16, 26). In most of these studies, statin therapy attenuated inflammation, steatosis, and fibrosis (8, 14, 16). Recently, a large multicenter study demonstrated in patients at risk for NASH that statin treatment protects, mainly in patients without the PNPLA3 I148M polymorphism, from the full spectrum of liver injury (6). Summarizing these facts, statins may not only decrease risk for the development of CVD, but may also improve NASH in certain NAFLD patients.

In previous studies, others and our group showed that statins decrease experimental fibrosis in rats (23, 43). Statins elicited these effects by inhibition of HSC activation and subsequent improvement of sinusoidal endothelial cell phenotype (17, 23). Additionally, HSC proliferation is inhibited when cells are treated with HMG-CoA reductase inhibitors (33). Furthermore, statin treatment has been shown to decrease portal pressure in human and experimental cirrhosis (1, 42). The beneficial effects on hepatic fibrosis and portal hypertension are mediated by the pleiotropic effects of statins, probably due to a large extent by downregulation of RhoA and upregulation of KLF2 (23, 42, 47). Furthermore, downregulation of RhoA may lead to increased expression of eNOS, enhancing the direct effects of SMV on RhoA (49). In this context, it is interesting that the

Fig. 5. Effect of GTPase inhibitors and simvastatin (SMV) on hepatic stellate cell activation. A and B: representative sections and quantification of αSMA histological staining showed a significant decrease of αSMA positive staining in apoE−/− NASH mice after SMV (inhibitor of RAC1, Ras and RhoA) treatment compared with control NASH mice (+ solvent). Treatment with NSC23766 (NSC; RAC1 inhibitor) and lethal toxin from Clostridium sordellii (LT; RAC1 and Ras inhibitor) showed only a trend to decreased αSMA positive staining. C: decreased gene expression of αSMA was observed only after SMV treatment. D: densitometric quantification of Western blot analysis showed significant decrease of αSMA and cathepsin D on protein level after treatment with SMV. Treatment with LT tendentially decreased αSMA and cathepsin D protein levels. Data are presented as means ± SD. The scale bar is 100 μm for αSMA staining. *P < 0.05 was considered significant.
small GTPases RAC1, Ras, and RhoA play an important role for the development of MS. Apart from lowering serum cholesterol levels, statins are able to inhibit these small GTPases downstream of G protein-coupled receptors (30, 31). To investigate the distinct role of each of these GTPases, different inhibitors were used: on the one hand NSC, a specific inhibitor of RAC1 (10) and LT (50), which inhibits RAC1 and Ras simultaneous at the same extent, and on the other hand SMV, an inhibitor of RAC1, Ras, and RhoA at the same time.

Interestingly, this study in NASH mice demonstrates that treatment with SMV beneficially affects fibrosis and inflammation, mainly because of the inhibition of RhoA- and Ras-mediated pathways. Thereby, the positive effects of SMV on hepatic inflammation seemed to be independent of the inflammasome and caspase activation. Importantly, the treatment with SMV was started after the mice had developed a significant liver damage, already after 1 wk of WD. Therefore, our approach mimics the clinical situation, treating patients with already developed NAFLD. Furthermore, the present study confirms our previously published observations that statins attenuate fibrogenesis due to direct effects on these pathways in activated HSCs (17, 36, 42, 43).

Inhibition of RAC1 and Ras by use of LT indicated a trend toward decreased fibrosis, while steatosis was not influenced. Therefore, RAC1 apparently plays no significant role in fibrogenesis, at least in this NASH model. The toxin LT is able to activate the inflammasome (27). However, activation of the inflammasome did not lead to decreased expression of Tnfα and Ccl2.

The present study also demonstrates that the inhibition of Ras/ERK1/2 pathway in combination with inhibition of RhoA/Rho kinase pathway is crucial for the antifibrotic and anti-inflammatory effects of statins. However, no impact of small GTPase inhibition could be assessed on steatosis, at least not in our animal model. Triglycerides were not assessed directly, but the staining for hepatic fat accumulation in the liver remained unchanged after statin application. Explanations for this finding might be the rather short statin treatment of only 6 wk, as well as the specific genetic background of the mice. The last hypothesis parallels the recent findings in humans, where genetic predisposition for NASH was not improved by statins (6).

Despite the described effects on hepatic steatosis, the effect of SMV on serum VLDL and LDL cholesterol reductions was less prominent compared with the effects of NSC and LT. We focused on the pleiotropic effects of SMV, which are cholesterol independent.

Similarities between the apoE−/− mice receiving a WD and human MS (37) render this model suitable for testing drugs or lifestyle changes for NASH treatment and/or prevention, as shown in the following figures:

Fig. 6. Inhibition of Ras/ERK and RhoA/Rho kinase signaling is responsible for the SMV effect on hepatic fibrosis. A–C: protein expression in apoE−/− NASH mice (+ solvent) and after treatment with SMV or GTPase inhibitors (NSC and LT). A and C: protein expression of RhoA signaling via Rho kinase. Treatment with simvastatin (SMV; inhibitor of RAC1, Ras, and RhoA) decreased expression of RhoA and downstream components. LT (RAC1 and Ras inhibitor) and NSC23766 (NSC; RAC1 inhibitor) showed no effect. B and C: protein expression of Ras downstream signaling via Ras/RAF1/ERK1/2. Protein expression of Ras, RAF1, and activity of ERK1/2 (pERK1/2) was decreased after SMV and partly after LT treatment. Data are presented as means ± SD. *P < 0.05 was considered significant.

G731 INHIBITION OF RhoA AND Ras IN NASH
AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00063.2016 • www.ajpgi.org
shown in our current study. Importantly, combined inhibition of Ras and RhoA should be further evaluated in NASH treatment.

In summary, statins are promising agents due to the multi-target effects for the treatment of NASH. We show here that SMV decreased inflammation and fibrosis due to inhibition of RhoA and Ras downstream signaling, whereas RAC1 inhibition showed no effect.

ACKNOWLEDGMENTS

The authors thank Gudrun Hack, Silke Bellinghausen, Anja Kerksee, and Mariana Rodrigues Davanso for excellent technical assistance.

GRANTS

Support for this study was provided by a grant from the Bonner Forum for Biomedicine (to J. Trebicka and S. Zimmer); Deutsche Forschungsgemeinschaft Grant SFB TRR57 P18 (to J. Trebicka); European Commission Directorate-General for Research and Innovation Grant 668031 (to J. Trebicka); Ernst-Bertha-Grimmel Stiftung Grant 6/15 (to J. Trebicka); and H.J. & W. Hector Stiftung Grant M60.2 (to J. Trebicka).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


