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Simvastatin Inhibits Epithelial-to-Mesenchymal Transition Through Induction of HO-1 in Cultured Renal Proximal Tubule Cells

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Abstract. *Background/Aim: Studies have shown that simvastatin (SIM) inhibits epithelial-mesenchymal transition (EMT), a key step in fibrosis, and activates the anti-fibrotic heme oxygenase-1 (HO-1) gene in renal proximal tubule cells independent of its lipid-lowering. We tested the hypothesis that SIM inhibits EMT via HO-1-dependent suppression of reactive oxygen species (ROS) release. Materials and Methods: Renal proximal tubule cells were treated with either 10 μM SIM or 10 ng/ml transforming growth factor-β1 (TGFβ1) or with their combination and promoter activity of the alpha-smooth muscle actin (α-SMA) gene, stress fiber formation (markers of EMT), as well as ROS production were determined. HO-1 was manipulated via genetic and pharmacologic means. Results: SIM prevented TGFβ1-dependent EMT and ROS production. Inhibition/knockdown of HO-1 reversed, while induction/overexpression of HO-1 emulated beneficial effects of SIM. Conclusion: SIM, via HO-1, suppresses TGFβ1-dependent ROS production and, hence, EMT. Further evaluation of the anti-fibrotic nature of SIM in the kidney would be useful in the treatment of chronic kidney disease.*

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are well-known for their cholesterol lowering, as well as cardioprotective properties (1, 2). Over the past several years, statins have been demonstrated to have protective effects in other organ systems, such as the liver (3), lung (4) and kidney (5), which are not dependent on lipid reduction. Simvastatin (SIM) is a well-studied statin. Multiple studies have demonstrated its anti-oxidant effects (5) and that SIM induces the heme oxygenase-1 (HO-1) enzyme (6), which has recently been implicated in a multitude of protective processes.

HO-1 is one of three isoforms of the heme oxygenase enzyme, which makes up the rate-limiting step in heme degradation. HO-1 is ubiquitous in mammalian cells and cellular expression of HO-1 is up-regulated in response to oxidative stress and cellular injury (7). In the kidney, *HO-1* protects the proximal tubules from adverse effects of oxidative stress that prevents onset of fibrosis (7). Conversely, HO-1-knockout mice exhibit an increase in the activity of the pro-fibrotic transforming growth factor-β1 (TGFβ1) and production of α-smooth muscle actin (α-SMA) (8), a marker of the epithelial to mesenchymal transition (EMT) (9), which plays a pivotal role in renal fibrosis associated with a wide range of kidney diseases (10).

The implication that statins could potentially be useful to slow or prevent chronic kidney disease (CKD) is significant as Medicare costs for CKD in 2010 were in excess of 40-billion dollars (11). Use of SIM to attenuate the EMT process has been demonstrated (12); however, the mechanism of attenuation is currently unknown. We recently demonstrated that SIM can induce HO-1 in cultured proximal tubule cells (13), which may play an important role in protection of the kidney from EMT and consequent fibrosis.

In the current study, we tested the hypothesis that SIM induces HO-1, which inhibits TGFβ1-dependent ROS production and subsequent EMT in renal proximal tubule cells.

Materials and Methods

Cell line and treatment. The porcine proximal tubule cell line LLC-PK1 was grown and maintained in DMEM (Life Technologies, Grande Island, NY, USA) containing 10% fetal bovine serum in a 5% CO_2 atmosphere. Cells were treated with 10 ng/ml TGFβ1 (R&D Systems, Minneapolis, MS, USA). In some experiments, cells were pre-treated with 10 μM SIM (Sigma-Aldrich, St. Louis, MO, USA) 12 h prior to treatment with TGFβ1. HO-1 activity/expression was inhibited with 10 μM tin-protoporphyrine (SnPP, Sigma-Aldrich, St.

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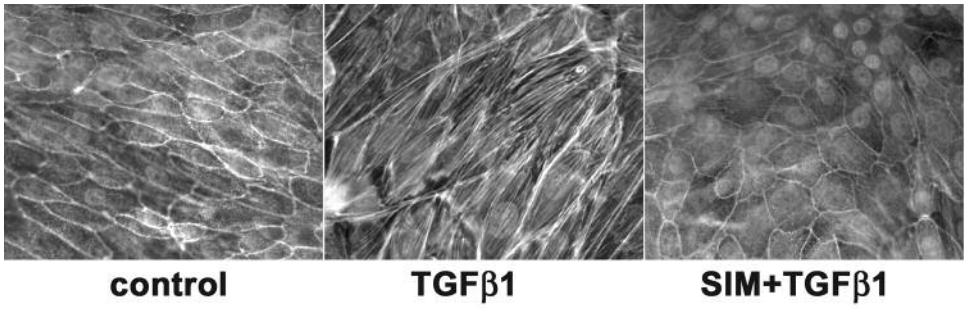

Figure 1. *Simvastatin prevents TGFβ1-mediated epithelial-mesenchymal transition in cultured renal proximal tubule cells. LLC-PK1 cells were pre-treated or not with 10 μM simvastatin (SIM) prior to 3-day-treatment with 10 ng/ml TGFβ1. Formation of F-actin fibers were visualized by phalloidin staining, as described in the Materials and Methods section. Pictures shown are representative of three independent experiments.*

Louis, MO, USA) or by transfection with an *HO-1* siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Endogenous HO-1 was induced by 20 μM cobalt-protoporphyrine (CoPP; Sigma-Aldrich, St. Louis, MO, USA) or ectopically overexpressed by transfecting an HO-1 plasmid (Dharmacon/Openbiosystems, Pittsburg, PA, USA). Production of ROS was inhibited by 10 μM N-acetyl-cysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA). The University of Mississippi Medical Center Institutional Biosafety Committee approved the use of this cell line.

Reporter luciferase studies. LLC-PK1 cells were cotransfected with either an HO-1-promoter luciferase (14) or an α-SMA-promoter luciferase (15) and renilla luciferase (Promega, Madison, WI, USA) plasmid using Lipofectamine 3000 per manufacturer suggestion (Life Technologies, Grand Island, NY, USA). A Dual Luciferase assay kit (Promega, Madison, WI, USA) was used to determine firefly (reporter luciferase) and renilla luciferase activity. Values were calculated as firefly/renilla ratios and expressed as percentage of the control values.

Reactive oxygen species (ROS) production. Cellular production of ROS was determined using the oxidant-sensitive 2',7'-dichlorofluorescein-diacetate (DCFDA; Invitrogen, Grand Island, NY, USA) as described elsewhere (16). ROS production was calculated as a percentage of untreated cells.

Phalloidin staining and fluorescence microscopy. F-actin fiber formation was visualized by staining of cultured cells with Alexafluor 488 phalloidin (Invitrogen, Grand Island, NY, USA) as described elsewhere (17). Fluorescence was observed with a Nikon Eclipse TS100F (Nikon Instruments Inc, Lewisville, TX, USA) inverted microscope equipped with a DAPI, FITC or CY3 filter at 400x magnification. Images were captured by a Nikon DS cooled camera (Nikon Instruments Inc.) and analyzed with the NIS Elements Basic Research 3.0 software (Nikon Instruments Inc.).

Statistical analysis. All studies consisted of continuous variables that were displayed using mean plus standard deviations. Differences between groups were determined using one-way ANOVA with the Holm-Sidak *post-hoc* test. Significance is reported for $p<0.05$. Analyses were completed using SigmaStat 3.5 software (Systat, San Jose, CA, USA).

Results

SIM inhibits TGFβ1-mediated EMT. During EMT, epithelial cells exhibit increased stress fiber formation. Accordingly, LLC-PK1 cells were treated with 10 ng/ml TGFβ1 for 3 days in the presence or absence of 10 μM SIM. As seen in Figure 1, control cells exhibited strong peripheral F-actin staining (bright staining) with rare occurrence of central stress fibers. In contrast, TGFβ1 treatment decreased marginal F-actin staining with appearance of strong stress fibers, that is consistent with the onset of EMT. Also, the cells lost their original "cobblestone" morphology and acquired fibroblast-like shape (data not shown). Importantly, pretreatment with SIM prevented formation of stress fibers.

SIM inhibits TGFβ1-mediated induction of the α-SMA promoter. To determine if SIM inhibits TGFβ1-mediated EMT, LLC-PK1 cells were co-transfected with plasmids containing an α-SMA promoter luciferase reporter, as well as a renilla luciferase. Cells were then treated with either 10 ng/ml TGFβ1 or 10 μM SIM alone or with SIM 12 h prior to treatment with TGFβ1. After 24 h, luciferase activities were determined. As is seen in Figure 2, treatment with TGFβ1 resulted in significant induction of the α-SMA promoter, which was prevented by pretreatment with SIM. These results imply that SIM attenuates TGFβ1 induction of α-SMA and, hence, the EMT.

TGFβ1-mediated induction of the α-SMA promoter is ROS-dependent. Next, we set out to determine if induction of the α-SMA promoter *via* TGFβ1 is a ROS-dependent process. To determine this, we co-transfected LLC-PK1 cells with plasmids containing an α-SMA promoter luciferase reporter, as well as a renilla luciferase. Cells were then treated with either 10 ng/ml TGFβ1 or with 10 μM N-acetyl-Cysteine (NAC) 30 min prior to treatment with TGFβ1. After 24 h, luciferase activities were determined. As is shown in Figure 2, NAC significantly

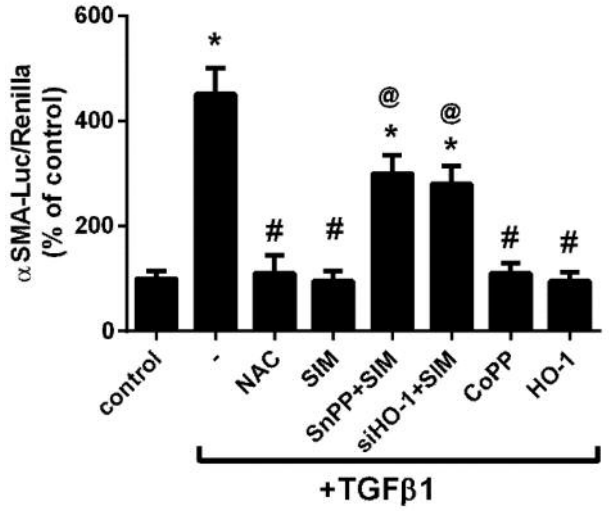

Figure 2. *Oxidative stress and HO-1 modulates TGFβ1-dependent activation of the α-SMA promoter in cultured renal proximal tubule cells. LLC-PK1 cells were co-transfected with plasmids containing the α-SMA promoter luciferase reporter, as well as a plasmid containing a renilla luciferase and treated with 10 ng/ml TGFβ1 for 24 h. Some cells were pre-treated with either 10 μM NAC or 10 μM SIM prior to treatment with TGFβ1. Some cells were also pre-treated with 10 μM SnPP for 30 min or transfected with an HO-1 siRNA (siHO-1) prior to treatment with SIM+TGFβ1. Another set of cells were pre-treated with 20 μM CoPP or transfected with an overexpressing plasmid prior to treatment with TGFβ1. α-SMA/renilla luciferase activities were recorded after 24-hours. n=3, *p<0.05 compared to control; #p<0.05 compared to TGFβ1-treated; @p<0.05 compared to SIM+TGFβ1-treated.*

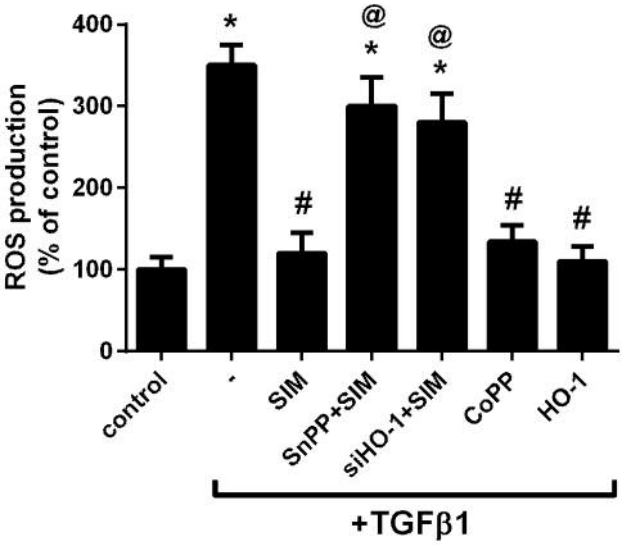

Figure 3. *SIM attenuates TGFβ1-dependent ROS production via HO-1 in cultured renal proximal tubule cells. LLC-PK1 cells were treated with 10 μM SIM overnight and 10 ng/ml TGFβ1-dependent ROS production was determined. In some experiments, cells were pre-treated with 10 μM SnPP or transfected with an HO-1 siRNA prior to treatment with SIM+TGFβ1. Some cells were treated with 20 μM CoPP or transfected with an HO-1-expressing plasmid prior to treatment with TGFβ1. n=3; *p<0.05 compared to control; #p<0.05 compared to TGFβ1-treated; @p<0.05 compared to SIM+TGFβ1-treated.*

inhibited TGFβ1-mediated induction of the α-SMA reporter. This demonstrates that induction of α-SMA by TGFβ1 is dependent on ROS production.

SIM attenuates TGFβ1-dependent activation of the αSMA promoter via HO-1. Earlier we have reported that SIM activates the HO-1 gene in cultured renal proximal tubule cells (13). Hence, we tested the hypothesis that SIM-mediated induction of HO-1 attenuates TGFβ1-dependent activation of the α-SMA promoter and, hence, EMT. To determine this, LLC-PK1 cells were co-transfected with an α-SMA promoter luciferase reporter and a renilla luciferase plasmid. Cells were either pre-treated with 10 μM SnPP or transfected with an *HO-1* siRNA (to inhibit HO-1) prior to treatment with 10 μM SIM followed by 10 ng/ml TGFβ1. Figure 2 shows that inhibition of HO-1 abolishes beneficial effects of SIM on TGFβ1-mediated α-SMA activation. In contrast, activation of HO-1 (*via* treatment of 20 μM CoPP or ectopic HO-1 overexpression) mitigates TGFβ1-mediated α-SMA activation, similar to pre-treatment with SIM. These experiments prove that SIM inhibits TGFβ1-mediated α-SMA activation *via* induction of HO-1.

SIM attenuates TGFβ1-dependent induction of the α-SMA promoter via inhibiting ROS production. Results in Figure 2 demonstrate that TGFβ1-mediated activation of α-SMA promoter is ROS-dependent. Hence, we tested whether SIM attenuates TGFβ1-associated ROS production in order to inhibit α-SMA induction. Accordingly, LLC-PK1 cells were treated with 10 μM SIM overnight and, then, TGFβ1-dependent ROS production was determined as described in the Materials and Methods section. Figure 3 shows that TGFβ1 significantly increased ROS production, which was significantly reduced in the presence of SIM.

SIM inhibits TGFβ1-dependent ROS production via HO-1. Previously we demonstrated that SIM attenuates ROS production *via* induction of HO-1 in renal proximal tubule cells (13); therefore, in the current study we set out to determine if SIM inhibits TGFβ1-dependent induction of α-SMA through HO-1-dependent inhibition of ROS release. Accordingly, LLC-PK1 cells were pretreated with 10 μM SnPP or transfected with an *HO-1* siRNA (siHO-1) then treated with SIM for 12 h and TGFβ1-dependent ROS production was determined. Figure 3 demonstrates that inhibition of HO-1 (*via* SnPP or siHO-1) blunted beneficial effects of SIM on TGFβ1-dependent ROS production. In

contrast, endogenous activation of HO-1 by 20 μM CoPP or ectopic overexpression of HO-1 mitigated TGFβ1-dependent ROS production similar to SIM treatment. These results imply that SIM attenuates TGFβ1-mediated ROS production *via* HO-1 induction.

Discussion

CKD is associated with the deposition of extracellular matrix (ECM) throughout the kidney tubules, eventually leading to complete failure of the kidneys (18). The EMT is thought to be responsible for renal interstitial fibrosis and consequent progression of CKD and deposition of ECM (10). Progression of CKD can be slowed with the use of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (19). To date however, there have been no treatments that can stop or reverse the progression of the CKD towards end-stage. Given the significant cost burden that renal disease has (11), there is significant incentive to determine the underlying molecular process and potential treatments to stop progression of CKD.

There are several signaling pathways believed to be involved in the EMT process; however, the TGFβ1 pathway is believed to be the primary pathway through which all other pathways interact. Here we show that TGFβ1 indeed induces EMT in cultured renal proximal tubule cells as demonstrated *via* formation of stress fibers (Figure 1) and induction of the a-SMA promoter (Figure 2). Downstream activation from TGFβ1 signaling is heavily reliant on ROS production (20). Conversely, ROS scavengers can block EMT in renal tubular cells (20). Similar results were demonstrated in our current study that demonstrates the requirement of ROS in the process of EMT (Figure 2) as the scavenger NAC significantly attenuated TGFβ1-dependent induction of the α-SMA promoter.

HO-1 is an enzyme ubiquitous in mammalian cells and known to have anti-oxidant properties (7). Due to its ubiquitous nature and cytoprotective activity, HO-1 has become a target of interest in the clinical medicine (21). The importance of HO-1 in counter-acting the activity of TGFβ1 during kidney injury has been observed in *HO-1* knockout mice, which, in the presence of TGFβ1, demonstrate drastic increases in renal fibrosis (8). Our current study further supports the protective role of HO-1 in the process of EMT: activation of the endogenous HO-1 or its ectopic overexpression attenuated TGFβ1-dependent production of ROS and activation of α-SMA, while inhibition of HO-1 (*via* SnPP or *HO-1* siRNA) exacerbated it (Figures 2 and 3). To our knowledge, however, the mechanism through which treatment with SIM produces an anti-fibrotic effect in the kidney tubules has yet to be determined. In the present study, we were able to identify that SIM's anti-fibrotic effect was directly related to its induction of HO-1 and consequent inhibition of TGFβ1-mediated ROS production in cultured renal proximal tubule cells (Figures 2-3).

Since meta-analysis has shown the beneficial effects of high-intensity statins as renal protective agents in CKD (22), our data may help identify molecular targets for statin-treatment to ameliorate progression of CKD in the renal patient.

Acknowledgements

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