Low simvastatin concentrations reduce oleic acid-induced steatosis in HepG₂ cells: An \textit{in vitro} model of non-alcoholic fatty liver disease

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Abstract. Non-alcoholic fatty liver disease (NAFLD) is an inflammatory condition caused by hepatic lipid accumulation that is associated with insulin resistance, diabetes and metabolic syndrome. Although statins should be used with caution in liver diseases, they are increasingly investigated as a possible treatment for NAFLD. The present study recreated an \textit{in vitro} model of NAFLD using HepG₂ cells exposed to oleic acid (OA), which was used to quantify OA-induced lipid accumulation in HepG₂ cells treated with various concentrations of simvastatin. In addition, the effect of simvastatin on HepG₂ cell morphology and microparticle generation as a marker of cell apoptosis was assessed. OA-induced lipid accumulation was quantified by Oil Red O staining and extraction for optical density determination. Stained lipid droplets were visualized using phase contrast microscopy. Furthermore, HepG₂ cell-derived microparticles were counted by flow cytometry subsequent to staining for Annexin V. HepG₂ cells treated with 0-1 mM OA showed dose-dependent lipid accumulation. Treatment of HepG₂ cells with increasing concentrations of simvastatin followed by treatment with 1 mM OA showed that low simvastatin concentrations (4-10 µM) were able to reduce lipid accumulation by ~40%, whereas high simvastatin concentrations (20 and 30 µM) induced apoptotic changes in cell morphology and increased the production of Annexin V⁺ microparticles. This suggests that low simvastatin doses may have a role in preventing NAFLD. However, further investigations are required to confirm this action \textit{in vivo} and to determine the underlying mechanism by which simvastatin reduces hepatic steatosis.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; HepG₂, human hepatocellular carcinoma cell line

Key words: non-alcoholic fatty liver disease, oleic acid, simvastatin, steatosis, microparticles

Introduction

The liver is a vital organ that is highly susceptible to fat accumulation, resulting in a condition known as fatty liver disease or hepatic steatosis (1). Although chronic alcohol consumption is a major cause of fatty liver disease (2), non-alcoholic fatty liver disease (NAFLD) is also common and is strongly associated with central obesity, insulin resistance, hyperlipidemia and the metabolic syndrome (3). Insulin resistance causes increased lipolysis and thus leading to high levels of plasma free fatty acids (FFAs), as well as increased FFA uptake by hepatocytes, which results in the formation of intracellular lipid droplets (4). Hepatic lipid accumulation can progress from simple steatosis to non-alcoholic steatohepatitis (NASH), which includes hepatocellular injury, inflammation and fibrosis (5). In addition, further severe complications may occur, such as liver cirrhosis and hepatocellular carcinoma (6).

In order to prevent and treat NAFLD, lifestyle changes including weight reduction and increased physical activity are considered as the first-line approach (7). Although pharmacologic therapy is mainly directed toward increasing insulin sensitivity using insulin-sensitizing medications, such as metformin and pioglitazone (8), the use of other medications is increasingly investigated. Medications used to treat dyslipidemia, such as gemfibrozil (a triglyceride lowering agent) and statins (HMG-CoA reductase inhibitors that reduce cholesterol synthesis in the liver), are among the agents investigated (9). Gemfibrozil has been found to be beneficial in the treatment of patients with NASH as it was able to significantly reduce the elevated levels of hepatic aminotransferases when compared with the control group (10). Similarly, atorvastatin was efficacious in the treatment of patients with NAFLD and dyslipidemia as it was effective in reducing hepatic aminotransferases and improving lipid profile (11,12). Although statins are administered with caution in patients with elevated aminotransferases due to the risk of statin-induced hepatotoxicity, this concern is not clinically important as statin-associated hepatic adverse effects are of low incidence, reversible and dose-dependent (13). Thus, statins should not be contraindicated in patients with NAFLD and elevated liver enzymes as they are promising medications for these conditions (9).

Through a review of the relevant literature using PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and English as a
search language, the majority of studies were found to clinically evaluate the effect of statin use on reducing the elevated hepatic enzyme levels in patients with NAFLD (14-17).

A previous study evaluated the effect of statin therapy on hepatic lipid accumulation by comparing the liver density measured by computerized tomography prior to and following statin use for a certain period of time (12). Changes in the density reflected alterations in hepatic lipid accumulation in response to statin therapy. However, to the best of our knowledge, the efficacy of statins in reducing hepatic intracellular lipid accumulation has not been previously assessed in vivo. Therefore, the aim of the present study was to evaluate the effect of simvastatin on hepatic intracellular lipid accumulation on an in vitro model of NAFLD. A human hepatocellular carcinoma cell line (HepG2) was exposed to oleic acid (OA), which is a monounsaturated omega-9 fatty acid, and this served as a model of NAFLD (18,19). Specifically, the study aimed to visualize and quantify OA-induced lipid accumulation in HepG2 cells treated with various concentrations of simvastatin.

Materials and methods

Cell line. HepG2 cells were a gift of Professor David Morris at the Department of Surgery at St. George Hospital Clinical School (New South Wales, Australia). The cell line was originally obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK).

Cell culture method. HepG2 cells were maintained in Dulbecco's modified Eagle's medium that contained 2 mM L-glutamine (both purchased from Lonza Australia Pty., Ltd., Mount Waverley, Australia) and 4.5 g/l glucose (Sigma-Aldrich, Castle Hill, Australia), and was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 0.1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 24 mM sodium hydrogen carbonate and 25 mM HEPES [also known as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (both purchased from Lonza Australia Pty., Ltd.). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air (v/v). Cell growth and induction of cell death was monitored by phase contrast microscopy (Olympus CKS; Olympus Corporation, Tokyo, Japan) and by detecting the generation of HepG2 cell-derived Annexin V⁺ microparticles. Digitized images were generated using a SPOT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) using SPOT version 2.1.2 software.

Cell culture treatments. HepG2 cells were grown in 96-well plates at a density of 1x10⁴ cells/well until ~70% confluence was reached. Next, cells were deprived from FBS for 24 h before treatment with 0-10, 20 and 30 µM simvastatin (Sigma-Aldrich). Oleic acid (OA; Sigma-Aldrich) was dissolved at a concentration of 12 mM in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl and pH 7.4) that contained 11% fatty acid-free bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA, USA) by sonication at a frequency of 10 KHz with two 5 min pulses (Soniprep 150 fitted with an exponential probe; Thermo Fisher Scientific, Inc.) prior to shaking at 37°C for 15 h (20) using an OM10 Orbital Shaking Incubator (Ratek Instruments Pty., Ltd., Boronia, Australia). OA solution was filtered using a 0.22 µm filter and stored at 4°C prior to use. HepG2 cells were treated with increasing concentrations of OA solution (0-1 mM) for 24 h to determine the optimal concentration that induces cellular OA accumulation. To determine the effect of simvastatin on OA-induced HepG2 cell steatosis, cells were treated with increasing concentrations of simvastatin (0-30 µM) for 24 h before treatment with 1 mM OA. Simvastatin was activated prior to use with NaOH as previously described (21), and according to the manufacturer's instructions.

Oil Red O staining. A stock solution of 0.35% Oil Red O (BDH Chemicals, Poole, England) in isopropanol was prepared, filtered twice using a 0.22 µm filter and diluted in double-distilled H₂O (ddH₂O: 3:2) prior to use. To detect and quantify cellular lipid accumulation, OA-treated HepG2 cells were gently washed with PBS and fixed using 4% paraformaldehyde for 1 h at room temperature (RT). Subsequently, the cells were washed twice using ddH₂O and stained with Oil Red O solution for 20 min at RT. In order to remove the background staining, the cells were washed for 5 min with 60% isopropanol solution. Lipid droplet accumulation was detected by watching under the microscope. To quantify intracellular lipid accumulation, Oil Red O stain was extracted using pure isopropanol and the optical density was detected at 510 nm using a Spectramax 250 Plate reader; data analysis was performed using SoftMax Pro version 5.0 software (both purchased from Molecular Devices (UK), Ltd. (Wokingham, UK).

Microparticle quantification. HepG2 cell-derived microparticles were stained and quantified as previously described (22,23). The technique was performed according to the guidelines established by the International Society of Thrombosis and Haemostasis on the standardization of platelet-derived microparticle enumeration by flow cytometry (24), along with modifications suggested in the manufacturer instructions of the BD FACScanto flow cytometer (BD Biosciences, San Jose, CA, USA). The flow cytometer was calibrated to set the lower microparticles' detection limit as previously described (22). HepG2 cells in culture media were centrifuged at 400 x g for 5 min at room temperature to remove cellular debris. Subsequently, 10 µl samples were incubated at RT for 30 min with Annexin V-APC (eBioscience, Inc., San Diego, CA, USA) to detect phosphatidylserine microparticle expression as a marker of vesicles derived from apoptotic cells. Appropriate dilutions were made using calcium-rich binding buffer as supplied by Annexin V-APC manufacturer. Absolute microparticle numbers were also determined as previously described (22) using TruCount counting tubes (BD Biosciences).

Statistical analysis. Data for continuous variables are expressed as the mean ± standard deviation of three independent experiments for each treatment. Data was analyzed using the Student's t-test (SPSS version 18.0; IBM SPSS, Amronk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

OA induces dose-dependent HepG2 cellular lipid accumulation. High levels of plasma FFAs are implicated in the pathogenesis of NAFLD as they can accumulate in hepatocytes to form lipid droplets (4). This condition was recreated in vitro by treating HepG2 cells with increasing concentrations of OA (0-1 mM). Intracellular lipid droplets were negatively stained for Oil Red O dye in the OA-untreated HepG2 cells (Fig. 1A) and positively stained in the OA-treated cells (Fig. 1B). As shown in Fig. 1C, lipid accumulation in OA-treated cells was dose-dependent and the concentration of 1 mM OA was considered as an optimal concentration for the induction of lipid accumulation in HepG2 cells as a model of NAFLD.

Low simvastatin concentrations reduce OA-induced steatosis in HepG2 cells. To evaluate the effect of simvastatin on HepG2 cellular lipid accumulation, cultured HepG2 cells were exposed to increasing concentrations of simvastatin (0-10 µM) for 24 h before treatment with 1 mM OA. Subsequently, Oil Red O staining and extraction for optical density determination were performed in order to quantify the OA accumulation intracellularly. Fig. 2 shows that simvastatin was able to reduce OA accumulation in HepG2 cells in a dose-dependent manner over a concentration range of 1-4 µM. Further increase in simvastatin dose (up to 10 µM) was not able to reduce OA accumulation more than ~40%. This effect was also confirmed by visualizing Oil Red O staining using phase contrast microscopy (data not shown).

High simvastatin concentrations induce the release of HepG2 cellular microparticles and morphological characteristics of cell apoptosis. Microparticles are small (<1 µm in diameter) vesicles that originate from the plasma membranes of cells undergoing apoptosis (25). To evaluate the effect of simvastatin on HepG2 cell growth and induction of cellular apoptosis, cultured HepG2 cells were treated with low-dose (4 µM) and high-dose (20 and 30 µM) simvastatin for 24 h.
and compared with the untreated control (0 µM simvastatin; Fig. 3A-E). Samples from cell culture media were analyzed by flow cytometry to count the number of Annexin V+ microparticles released by the HepG2 cells. As shown in Fig. 3, low-dose simvastatin (4 µM) did not induce any change in cell morphology (Fig. 3B) and did not induce an increase in the number of Annexin V+ microparticles compared with that in the control group (Fig. 3E). By contrast, treatment high simvastatin doses (20 and 30 µM; Fig. 3C and D, respectively) induced changes in cellular morphology and significantly increased the generation of cell-derived Annexin V+ microparticles compared with the control (P<0.05; Fig. 3E). As revealed by phase contrast microscopy, HepG2 cells treated with 20 or 30 µM simvastatin prior to treatment with 1 µM OA showed reduced staining for Oil Red O compared with cells treated with 4 µM and the control cells (Fig. 3A-D).

**Discussion**

OA-induced lipid accumulation in HepG2 cells may act as an *in vitro* model for studying NAFLD. Consistent with other studies (18,26), the results of the present study demonstrated that lipid accumulation in HepG2 cells in response to OA treatment was dose-dependent and can be easily quantified using an Oil Red O colorimetric technique. This technique is based on the staining of intracellular lipid droplets by Oil Red O, followed by stain extraction and measurement of optical density that is proportional to the intracellular lipid content. In agreement with the findings of Cui et al. (18), the present study also revealed that HepG2 intracellular lipid quantification using the aforementioned staining technique provided reliable measurements of intracellular lipid-droplet levels. Assay results were able to reflect the dose-dependent uptake of OA and appeared entirely consistent with lipid droplets stained with Oil-Red O.
as visualized using microscopy. In addition, intracellular Oil Red O-stained lipid droplets can be directly visualized using phase contrast microscopy. Therefore, quantification of OA-induced HepG₂ cell steatosis may act as a valuable model to study the pathogenesis of NAFLD and assess the effect of possible treatments for hepatic steatosis.

In the current study, pretreatment of HepG₂ cells with low simvastatin doses (4-10 µM) was able to reduce the OA-induced intracellular lipid accumulation by ~40%. By contrast, pretreatment with high simvastatin doses (20 and 30 µM) induced HepG₂ cellular apoptosis that was detected by changes in cell morphology and production of cell-derived Annexin V⁺ microparticles. To the best of our knowledge, the present study quantified fatty acid-induced lipid accumulation in human hepatocellular carcinoma cells in response to simvastatin treatment for the first time. Previous similar studies have clinically assessing the efficacy of statin therapy in the treatment of patients with NAFLD by evaluating their effect on reducing elevated hepatic enzymes including aminotransferases and γ-glutamyl transferase (14-17). Recently, de Keyser et al have also shown that statin therapy for >2 years was associated with a lower prevalence of hepatic steatosis among overweight subjects (27). This protective effect was considered to be associated with the ability of statins to improve the lipid profile by inhibiting the HMG-CoA reductase pathway and by acting as anti-inflammatory, anti-oxidant and immune-modulatory agents (27-29). However, no previous study has shown any direct effect for statins on hepatic lipid accumulation at the cellular level. At the organ level, statins, which can reduce elevated serum triglyceride concentrations (30), may decrease hepatic lipid accumulation in overweight and obese subjects by reducing the serum levels of triglycerides and fatty acids (4).

Using an in vitro model of NAFLD to quantify cellular lipid accumulation in response to simvastatin treatment is considered the main strength of the current study in comparison with other clinical studies. Although the mechanism through which simvastatin can reduce intracellular HepG₂ lipid accumulation was not determined in the current study, this model can be used in the future to study possible mechanisms or to investigate other possible treatments for NAFLD. However, there are certain limitations in the present study. Using HepG₂ cells to investigate the effect of simvastatin or other HMG-CoA reductase inhibitors on intracellular lipid accumulation may be inappropriate or should be used carefully, since certain previous studies have demonstrated that simvastatin can induce growth inhibition and apoptosis in HepG₂ cells (31,32). However, this effect was dependent on the concentration of simvastatin and the duration of cell exposure to simvastatin. For instance, Huang et al (31) and Kah et al (32) have shown that treatment of HepG₂ cells with 8 or 10 µM simvastatin for 72 h induced ~50% decrease in cell viability. Huang et al (31) have also shown that lower simvastatin doses (2 and 4 µM) for shorter period of time (24 h) induced ~20% decrease in HepG₂ cell viability. In the current study, HepG₂ cell viability in response to simvastatin treatment was not measured as an indicator of cell apoptosis; by contrast, the number of Annexin V⁺ microparticles in the culture media was detected as an indicator of cell apoptosis, beside examining the cell morphology under a microscope as shown in Fig. 3. Furthermore, treatment of HepG₂ cells with low simvastatin dose (4 µM) for 24 h in the current study was able to reduce lipid accumulation by ~40% without increasing the number of Annexin V⁺ microparticles or changing cell morphology, which suggests that this dose reduces lipid accumulation without inducing cell apoptosis. However, treatment of HepG₂ cells with high doses of simvastatin (20 and 30 µM) for 24 h was found to induce increased generation of Annexin V⁺ microparticles and changes in cell morphology that were suggestive of cell apoptosis (Fig. 3). These results are consistent with the findings of Relja et al (33), which showed that HepG₂ cell apoptosis was induced by high doses of simvastatin (32 and 64 µM).

Determining the underlying mechanism by which low simvastatin concentrations can reduce HepG₂ intracellular lipid accumulation was out of the scope of the current study. However, more research is required to determine how simvastatin can produce this effect and whether other statins have similar effects on hepatic lipid accumulation. Possible roles for statins that deserve further investigations include their effect on fatty acid uptake by hepatic cells and their effect on hepatic intracellular triglyceride formation, which is stored as lipid droplets.

In conclusion, the present study demonstrated that low simvastatin concentrations can reduce HepG₂ intracellular OA-induced lipid accumulation without inducing cell death, whereas high simvastatin concentrations induced HepG₂ cell apoptosis, as revealed by detecting HepG₂ cell-derived Annexin V⁺ microparticles and changes in cell morphology. These findings support the results of previous clinical studies that encourage administration of statin therapy for the prevention of NAFLD (14-17). Additionally, the current study has shown that OA-treated HepG₂ cells may act as a model for studying other possible treatments for NAFLD.

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