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Simvastatin inhibits the core promoter of the *TXNRD1* gene and lowers cellular TrxR activity in HepG2 cells

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ABSTRACT

Thioredoxin reductase 1 (TrxR1) is a selenocysteine-containing redox-active enzyme that is thought to be important during carcinogenesis. We have recently shown that treatment with statins, HMGCoA reductase inhibitors, reduces the levels of TrxR1 in liver of both rat and human. The reduced TrxR1 levels were correlated with inhibited hepatocarcinogenesis in a rat model. The aim of the present study was to investigate if statins affect the activity of the human *TXNRD1* core promoter, which guides expression of TrxR1, and if the effects by statins on TrxR1 expression in liver could be reproduced in a cellular model system. We found that simvastatin and fluvastatin decreased cellular TrxR activity in cultured human liver-derived HepG2 cells with approximately 40% ($p < 0.05$). Simvastatin, but not fluvastatin or atorvastatin, also reduced the *TXNRD1* promoter activity in HepG2 cells by 20% ($p < 0.01$). In line with this result, TrxR1 mRNA levels decreased with about 25% in non-transfected HepG2 cells upon treatment with simvastatin ($p < 0.01$). Concomitant treatment with mevalonate could not reverse these effects of simvastatin, indicating that other mechanisms than HMGCoA reductase inhibition was involved. Also, simvastatin did not inhibit sulforaphane-derived stimulation of the *TXNRD1* core promoter activity, suggesting that the inhibition by simvastatin was specific for basal and not Nrf2-activated TrxR1 expression. In contrast to simvastatin, the two other statins tested, atorvastatin or fluvastatin, did not influence the TrxR1 mRNA levels. Thus, our results reveal a simvastatin-specific reduction of cellular TrxR1 levels that at least in part involves direct inhibitory effects on the basal activity of the core promoter guiding TrxR1 expression.

1. Introduction

Mammalian thioredoxin reductase (TrxR) is a selenocysteine-containing enzyme with a broad substrate specificity that is involved in many different cellular processes including defense against oxidative stress and support of DNA-synthesis [1,2]. There are three different isoforms of human TrxR; cytosolic TrxR1 encoded by *TXNRD1*, mitochondrial TrxR2 encoded by *TXNRD2* and TGR encoded by *TXNRD3*, with the latter isoform mainly being found in testis [3].

The levels of TrxR1 are often increased in cancer cells [4]. TrxR1, but not TrxR2, is increased in preneoplastic liver tissue (liver nodules) in a rat model for hepatocarcinogenesis [5] and we recently showed that treatment with statins (HMGCoA reductase inhibitors) reduces the levels of TrxR1 in both rat and human liver tissue [6]. Interestingly, there was a strong correlation between lower TrxR1 levels and inhibited carcinogenesis in the rat model, supporting the notion of an important role for TrxR1 during carcinogenesis [6]. In a mouse xenograft model using inoculation with lung cancer cells having knocked-down, TrxR1 it was also found by others that diminished levels of TrxR1 caused reduction in tumor progression, tumor size and formation of less metastases compared to mice injected with control cells [7]. These and several other findings (reviewed in [4]) collectively suggest that TrxR1 plays important roles during carcinogenesis. Thus, we speculated that the anticarcinogenic effects of statins, as described by us in the rat model of hepatocarcinogenesis [8], might be related to decreased levels of TrxR1 in the liver.

Abbreviations: TrxR, thioredoxin reductase; *TXNRD1*, human gene for TrxR1; SIM, simvastatin; FLU, fluvastatin; ATV, atorvastatin; ARE, antioxidant responsive element; Nrf2, NF-E2-related factor-2.

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Statins are cholesterol-lowering drugs that have protective effects in cardiovascular diseases [9]. Statins are today some of the most prescribed drugs in the western world. Possible anti-carcinogenic effects of statins have been reported in several epidemiological studies [10–12], although other studies challenge this notion [13,14]. There are also a number of reports suggesting that statins can have inhibitory effects on expression of selected target proteins. This includes inhibition of the keratin K6a promoter by simvastatin [15], simvastatin-mediated inhibition of CD40 expression through STAT-1alpha suppression [16] or inhibition of the PAI-1 promoter through down-regulated MEKK-1 activation [17]. However, simvastatin can also increase the activities of certain promoters, including that of PTEN [18], thereby illustrating gene specific effects. Here we therefore wished to study whether statins can trigger lower levels of TrxR1 in liver cells through effects on the core promoter in the TrxR1-encoding *TXNRD1* gene. This promoter lends the possibility of several levels of controls because it is a rather unique example of a strong Sp1-/Oct1-driven housekeeping type of promoter [19] combined with an Nrf2 responsive motif [20] that allows for induction of TrxR1 expression by stressors, such as oxidized LDL [21] or 4-hydroxynonenal [22]. Thus, regulation of TrxR1 expression may clearly be related to both carcinogenesis and atherosclerotic events, which in combination with the effects of simvastatin on TrxR1 expression warrants studies on any direct effects of simvastatin on *TXNRD1* core promoter activities.

2. Materials and methods

2.1. Cell culture

Human liver cancer HepG2 cells were cultured in MEM supplemented with 5% FCS, 1% penicillin/streptomycin, 1% L-glutamine and maintained in humidified atmosphere at 37 °C and 5% CO_2. No additional selenium source above that present in 5% FCS was added and the cells were thereby selenium starved but not selenium depleted [23]. Prior to statin exposure the HepG2 cells were split and plated in 24-well plates (for mRNA analysis) or 6-wells plates (for TrxR1 activity measurements) and pre-incubated for 2 days. Statins were diluted in dimethyl sulfoxide (DMSO) and added to the cells for 16–48 h at final concentration 1 μM. The non-treated controls were incubated with vehicle only. The experiments were performed in at least four independent experiments. For RNA experiments, the cells were harvested in Trizol (Invitrogen, UK) and kept at −80 °C. For TrxrR1 activity the cells were homogenized in 50 mM potassium phosphate, containing 1 mM EDTA, pH 7.4 and kept at −80 °C.

2.2. RNA preparation and cDNA synthesis

Total RNA extraction from HepG2 cells was performed using TRIzol according to manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA (0.5 μg) was reverse transcribed into cDNA with hexamer primer using high capacity cDNA synthesis kit (Applied Biosystems) according to the manufacturer's protocol. The cDNA was diluted ten times in water prior to real-time PCR analysis.

2.3. Real-time PCR

The mRNA levels of TrxR1 in human HepG2 cells and hepatocytes were determined by real-time PCR using gene-specific primers supplied by the manufacturer (Hs00182418_ml, Applied Biosystems). GAPDH was used as endogenous housekeeping control genes (P/N: 4310859E, Applied Biosystems). Quantitative real-time PCR was performed using the 7500 Fast Applied Biosystems. Reaction mixtures contained 2× Taqman reaction mix, TrxR1 Taqman Assay mix 1 μl cDNA template in a total volume of 15 μl. Thermal cycling conditions included activation at 95 °C (10 min) followed by 40 cycles each of denaturation at 95 °C (15 s) and annealing/elongation at 60 °C (1 min). Each reaction was performed in triplicate and non-template controls were included in each experiment. A vehicle treated sample was employed as a calibrator and the delta delta CT-formula was used as described elsewhere [24].

2.4. Promoter activity

The TrxR1 promoter construct were made using firefly luciferase reporter vector pGL3-Basic (Promega) as previously described [19]. As a negative control the pGL3 construct including the reversed sequence of TrxR1 promoter was used (denoted HB [19]).

The day before transfection, the cells were plated at the density of 4×10^4 HepG2 cells/well in 24-well plates. The TrxR1 promoter luciferase vector (1 μg) was co-transfected with 0.2 μg of pRL-TK reporter using Lipofecamine® LTX and Plus reagent (Invitrogen) with 2.5 μl of Lipofectamine and 0.5 μl of Plus reagent according to manufacturer's protocol. After 3 h the cells were exposed to statins (simvastatin, fluvastatin or atorvastatin at indicated concentrations) or solely vehicle (DMSO) over night (16 h). The cells were washed with phosphate-buffered saline and lysated in 100 μl of Passive Lysis Buffer (Promega). The firefly and Renilla luciferase activities were determined by the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instructions (Promega) in a single tube multimode reader for Luminescence (SDS Bioscience). The ratio between the TrxR1 promoter firefly luciferase signal and the control Renilla luciferase signal in the statin-exposed samples was compared to the ratios in the vehicle-treated samples.

2.5. TrxR activity

The TrxR activity in HepG2 cell homogenates was determined using a thioredoxin reductase assay kit from Cayman chemicals based upon measurement of gold-inhibited NADPH dependent DTNB reduction (item number 10007892). For this, HepG2 cells were homogenized in 50 mM potassium phosphate, containing 1 mM EDTA, pH 7.4 buffer and the activity per minute was calculated according to the manufactures manual and normalized against the total protein content as determined by Lowry [25]. The TrxR activity was analyzed from four independent experiments including duplicates at each time.

2.6. Statistical analysis

Statistical analysis of the mRNA expression and the activity was performed using GraphPad Prism software version 4.3 (San Diego, CA, USA). The enzyme activity, mRNA expressions and promoter activity of TxrR1 in statin-treated and vehicle treated groups in HepG2 were compared using the Mann–Whitney *U*-test, whereas the mRNA levels in hepatocytes were compared using Wilcoxon. Values of $p < 0.05$ were considered to be significant. Values are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. TrxR activity in HepG2 cells is lowered by simvastatin and fluvastatin

Simvastatin and fluvastatin treatment for 48 h resulted in an approximate 40% reduction of TrxR1 activity in the HepG2 cells compared to non-treated, control cells ($p = 0.03$). Treatment with

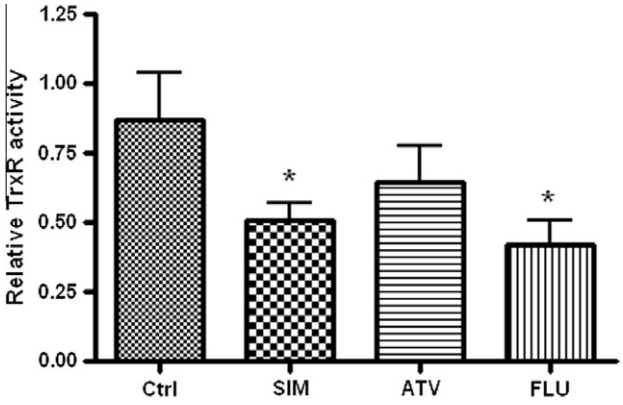

Fig. 1. Relative TrxR activity measured in whole lysates of HepG2 cells after 48 h of incubation with 1 μM of simvastatin (SIM), atorvastatin (ATV) or fluvastatin (FLU) compared to control (Ctrl) treated with solely vehicle (DMSO). $^{*}p < 0.05$; n.s. = b no significant difference ($p > 0.05$).

atorvastatin did not affect the TrxR-activity in a statistically significant manner (Fig. 1). Shorter treatment with simvastatin, fluvastatin or atorvastatin for 24 h gave no statistically significant difference in TrxR activity (data not shown).

3.2. TrxR mRNA levels in HepG2 cells are lowered by simvastatin

The influences of exposure of three different statins on the level of TrxR1 mRNA in HepG2 cells were investigated. The HepG2 cells were exposed to 1 μM of simvastatin, fluvastatin and atorvastatin for 16 h. When the cells were treated with simvastatin a significant 25% inhibition in mRNA level was observed ($p = 0.003$), whereas fluvastatin and atorvastatin did not affect the expression of TrxR1 mRNA in HepG cells under these conditions (Fig. 2). Statin exposure did not affect the endogenous control gene GAPDH.

3.3. Simvastatin inhibits the basal activity of the TXNRD1 core promoter in HepG2 cells

In order to study if the lower TrxR1 mRNA levels were related to altered promoter activity we next transfected HepG2 cells with a reporter construct including 0.8 kb of the *TXNRD1*-derived core

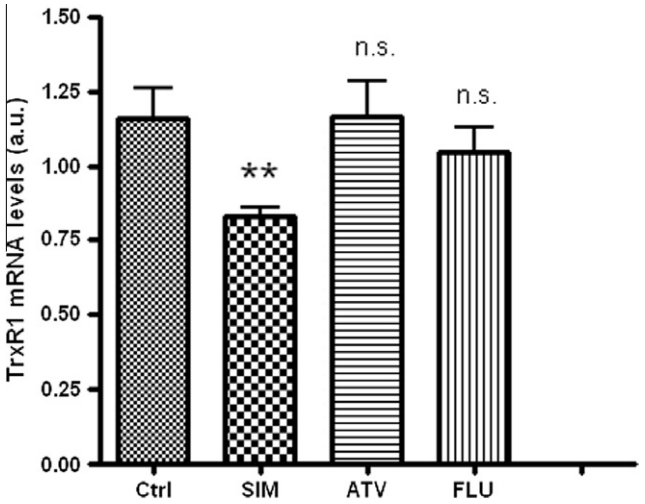

Fig. 2. TrxR1 mRNA levels (a.u. = arbitrary units; see Section 2) in HepG2 cells after overnight (16 h) of incubation with 1 μM of simvastatin (SIM), atorvastatin (ATV) or fluvastatin (FLU) compared to control (Ctrl) treated with solely vehicle (DMSO). $^{**}p < 0.01$; $^{*}p < 0.05$; n.s. = no significant difference ($p > 0.05$).

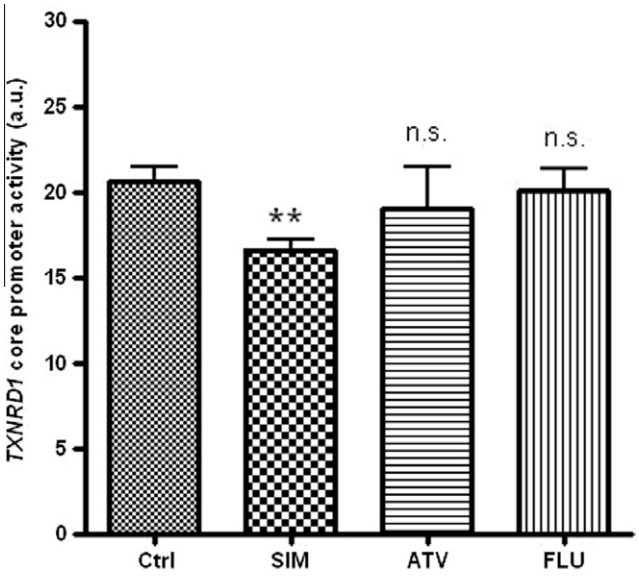

Fig. 3. *TXNRD1* core promoter activity (a.u. = arbitrary units; see Section 2) in luciferase reporter plasmid-transfected HepG2 cells after an overnight (16 h) incubation with 1 μM of simvastatin (SIM), atorvastatin (ATV) or fluvastatin (FLU) compared to control treated with vehicle (DMSO). $^{**}p < 0.01$; n.s. = no significant difference ($p > 0.05$).

promoter driving TrxR1 expression [19]. When cells were exposed to 1 μM simvastatin, about 20% inhibition in the basal promoter activity was observed ($p = 0.002$) (Fig. 3). In agreement with the mRNA levels in non-transfected cells (cf. Fig. 2) no alteration in promoter activity was observed for the two other statins tested (Fig. 3).

Cells were next treated with sulforaphane, a known Nrf2 inducer that was previously shown to activate the TrxR1 promoter activity about 4-fold in HepG2 cells [26], which induced the promoter about 3-fold in our conditions (Fig. 4A). Concordantly, we found that when simvastatin was added to these cells, no alteration in promoter activity was observed as the sulforaphane-triggered promoter activation remained (Fig. 4A). Thus, simvastatin inhibits the basal activity of the *TXNRD1* core promoter but not its activation by Nrf2.

In order to study if mevalonate could reverse the inhibitory effect of simvastatin, as mevalonate addition will by-pass the inhibitory effects of HMGCoA reductase inhibition, a high concentration of mevalonate (1 mM) was given to the cells. The result indicated that mevalonate itself induced the TrxR promoter activity (Fig. 4B) ($p = 0.03$). When the cells were co-administrated with simvastatin a significant decrease in promoter activity was again found ($p = 0.03$) (Fig. 4B), indicating that simvastatin exerted direct effects on the transcriptional activity of *TXNRD1* rather than indirect effects related to inhibition of the mevalonate pathway.

4. Discussion

Here we show that simvastatin treatment results in approximately 20–25% inhibition of *TXNRD1* basal promoter activity as well as TrxR1 mRNA levels in human liver HepG2 cells. In concordance with these findings, the total cellular TrxR activity was decreased with approximately 40% upon treatment with simvastatin.

The effect on the TrxR1 promoter activity, mediated by statins, could at least in part explain the decrease in TrxR1 levels in human liver tissue that we had previously found [6]. However, the reduced TrxR1 mRNA levels in human liver tissue [6] were much more pronounced (85% decrease) than found here with cultured HepG2 cells. This discrepancy between HepG2 cells and the *in vivo* data

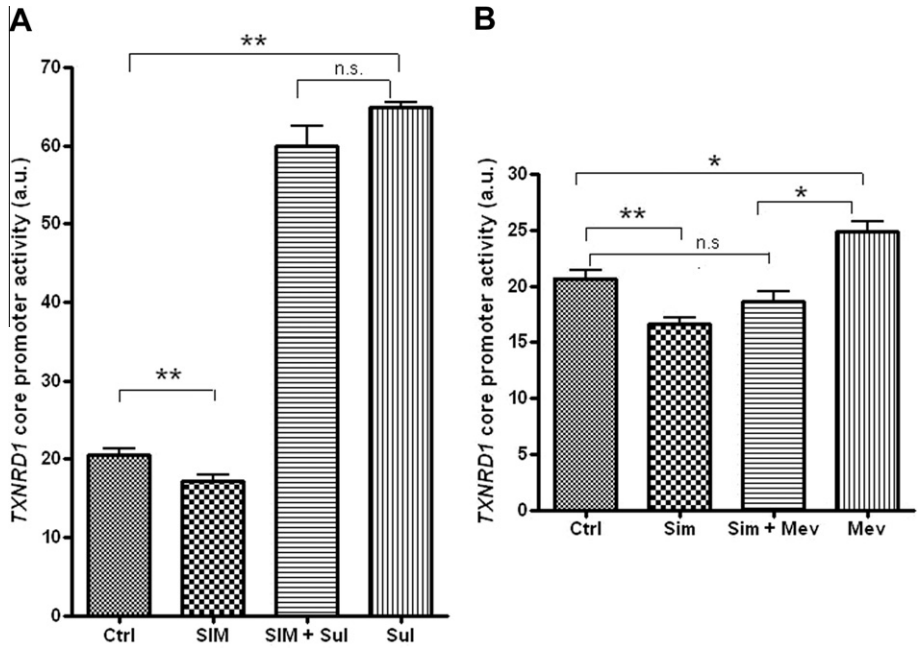

Fig. 4. (A) TrxR1 mRNA levels (a.u. = arbitrary units; see Section 2) in HepG2 cells exposed to vehicle (Ctrl), 1 μM simvastatin (SIM) with or without addition of 2 μM sulforaphane (Sul). $^{*}p < 0.05$; n.s. = no significant difference ($p > 0.05$). (B) Relative TrxR1 mRNA levels in HepG2 cells treated with vehicle (Ctrl), 1 μM simvastatin (SIM) with or without addition of 1 mM mevalonate (MEV). $^{**}p < 0.01$, $^{*}p < 0.05$; n.s. = no significant difference ($p > 0.05$).

might possibly be explained by the fact that the human subjects had been on statin medication for a long time whereas in the *in vitro* experiments presented here, the cells were exposed to statins only for 16–48 h. This could be considered as a "single-dose" of statin compared to the exposure in humans subjects with statin treatment for weeks to years [6]. Naturally, many mechanisms may contribute to larger effects on TrxR1 expression seen *in vivo* as compared to cell experiments. It is hereby clear, however, that simvastatin may exert inhibitory effects on the human *TXNRD1* promoter, which would likely contribute to the reduction of hepatic TrxR1 levels seen in patients subjected to simvastatin therapy.

With the basal activity of the *TXNRD1* core promoter requiring the house-keeping transcription factors Sp1 and Oct-1 [19] it seems likely that simvastatin modified the activities of these factors in HepG2 cells, as found here. This would also agree with prior findings that simvastatin can reduce the binding activity of Sp1 to the COL1A1 promoter in fibroblasts [27]. An important transcriptional regulation of TrxR1-gene is the antioxidative responsive element (ARE) in the 5′ region of the gene, activated by NF-E2-related factor-2 (Nrf2) [20,28]. Nrf-2 induction and subsequent induced transcription of TrxR1 in cell-experiments can thus be achieved by the treatment with sulforaphane [26] and it was interesting that statin treatment was here found not to affect the Nrf2-activation of the promoter but only its basal activity. This suggests that even though patients on statin treatment have lower steady-state levels of hepatic TrxR1, this could still retain the capacity of up-regulation upon events of hepatic oxidative stress (provided that our present findings in HepG2 cells replicates *TXNRD1* regulation *in vivo*).

Interestingly, simvastatin had significantly greater effects on TrxR1 expression and promoter activity in HepG2 cells as compared to atorvastatin and fluvastatin. All these statins are believed to have similar inhibitory effects on the HMGCoA reductase enzyme, so it is likely that simvastatin influenced the genetic regulation of TrxR1 independently of its HMGCoA reductase inhibition. This is further strengthened by the fact that addition of mevalonate did not reverse the effect. We have also observed that simvastatin, but not fluvastatin, inhibits the mRNA expression of TrxR1 in human arterial endothelial (HAEC) cells (unpublished results). Thus, simvastatin seems an especially active statin with regard to effects on TrxR1 expression, although we also found some inhibition on overall TrxR activity by fluvastatin. Since fluvastatin had no effects on either the *TXNRD1* promoter activity or the mRNA levels for TrxR1 it should have its effect(s) through other mechanisms. With regard to these findings it should be noted that statins were previously reported to have compound- and traget-specific effects on selenoproteins [29]. Specifically, using statin treatment of HepG2 cells it was found that GPx1 and GPx4 (but not TrxR) was post-translationally inhibited by atorvastatin, cerivastatin, and lovastatin [29]. The mechanism for post translational inhibition of specific selenoprotein activities by statins may be related to modulation of isopentenyladenosine modification of the tRNA for selenoysteine [28]. It has also been suggested that the effect on selenoprotein synthesis mediated by statins might explain some of the side effects of statins [30]. It should be of interest in future studies to analyze whether fluvastatin can inhibit expression of functional TrxR1 through modulation of the tRNA species used in its synthesis.

Chemically simvastatin is highly hydrophobic while atorvastatin and fluvastatin are more hydrophilic compounds [31]. Simvastatin is an inactive lactone prodrug that has to be metabolized to its active simvastatin-acid derivative, whereas fluvastatin and atorvastatin are administered in their pharmaceutically active forms. One might speculate that the different statins are taken up differently by the HepG2 cells. But we have shown that both simvastatin and fluvastatin are well absorbed in our cell-experiments and at concentrations used here (1 μM) the intracellular concentrations reach between 5 and 10 nM (unpublished data), close to the levels in human serum during therapy [32]. Further studies are warranted in order to elucidate the reason why simvastatin but not fluvastatin and atorvastatin affect promoter activity of TrxR1.

We conclude that at least part of the reduction of hepatic TrxR1 levels during statin treatment, as previously reported [6], is in part

due to a direct inhibition of the *TXNRD1* promoter activity, at least for simvastatin. However, it should be noted that the regulation of TrxR1 expression is complex and involves also post-transcriptional regulation on the mRNA-level [33] as well as modulation of tRNA species utilized for selenocysteine incorporation [28]. Such levels of regulation might explain the more pronounced decrease in TrxR activity as compared to the decrease in *TXNRD1* promoter activity. Even if the mechanisms and levels of regulation with regard to inhibitory effects of statins on TrxR1 expression in the liver are complex, these intriguing effects should clearly be considered with regard to the possible links between statin treatment and inhibition of carcinogenesis, as further discussed in Section 1.

Acknowledgments

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