

# **Statin-induced liver injury involves cross-talk between cholesterol and selenoprotein biosynthetic pathways**

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**Running title:** Statin-induced selenoprotein suppression

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Number of text pages:	30
Number of tables:	1
Number of figures:	6
Number of references:	39
Number of words in the abstract:	209
Number of words in the introduction:	616
Number of words in the discussion:	1052

**Abbreviations:** GPx, glutathione peroxidase; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; HO1, heme oxygenase 1; NQO, NAD(P)H:quinone oxidoreductase 1; ROS, reactive oxygen species; tBuOOH, *tert*-butyl hydroperoxide; TrxR, thioredoxin reductase.

## Abstract

Statins have become the mainstay of hypercholesterolemia treatment. Despite a seemingly clear rationale behind their use, the inhibition of HMG-CoA reductase, these compounds have been shown to elicit a variety of unanticipated and elusive effects and side-effects in vivo. Among the most frequently noted side-effects of statin treatment are elevations in liver enzymes. Here, we report our finding that atorvastatin, cerivastatin, and lovastatin at clinically common concentrations induce a selective, differential loss of selenoprotein expression in cultured human HepG2 hepatocytes. The primarily affected selenoprotein was glutathione peroxidase (GPx), whose biosynthesis, steady-state expression level, and catalytic activity were significantly reduced with 10 - 100 nM of the different compounds. Messenger RNA levels of GPx1 and GPx4 were unaffected by statin treatment, pointing at a post-transcriptional mechanism of selenoprotein suppression. Although statins at selenoprotein-modulatory doses were not cytotoxic by themselves, they yet induced a significantly increased sensitivity of the cells to peroxides, an effect that was largely reversible by supraphysiological concentrations of selenite. We conclude that statins inhibit the expression of inducible selenoproteins by preventing the mevalonate-dependent maturation of the single human selenocysteine-tRNA and may thereby evoke an increased vulnerability of the liver to secondary toxins. Selenoprotein modulation might constitute an important mechanism of statins to bring forth their clinical effects.

## Introduction

HMG-CoA-reductase inhibitors (statins) are specific and potent inhibitors of the rate-limiting step of cholesterol biosynthesis, namely the reduction of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to mevalonic acid (Tobert, 2003). Originally based on the cholesterol hypothesis of atherosclerosis, these compounds have been increasingly employed in the prevention and treatment of cardiovascular disease and various other pathologies of the circulatory system (Tobert, 2003; Topol, 2004; Wang et al., 2008), culminating in close to 30 million users in the US in 2005, based on prescription numbers (Stagnitti, 2008).

Despite their favorable overall safety profile, statins evoke a characteristic set of side-effects whose molecular origins have remained unsettled (Moosmann and Behl, 2004a; Baker, 2005; Bays, 2006; Jacobson, 2006; Argo et al., 2008). Mild-to-moderate elevations in liver transaminases are the most commonly seen side-effect of statin treatment in clinical practice, followed in frequency by muscular symptoms (Bays, 2006). While these elevations in liver enzymes usually remain asymptomatic, they do affect between 0.5% and 5% of all statin-treated patients in clinical studies, they occur with all statins, and they show a clear dependency on the statin dose administered (Bays, 2006), which has led to the recommendation by the FDA to monitor liver transaminases after initiation of therapy or dose increase (Jacobson, 2006). Notably, New Drug Application submissions indicate that mild increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to levels exceeding the upper limit of normal (ULN) by a factor of 2 occur in approximately 20% of statin-treated patients (Jacobson, 2006). Hence, elevations in transaminases constitute a regular response of statin-treated patients towards therapy with these drugs. Correspondingly, muscular side-effects of high-dosage statin medication are seen in approximately 10% of the patients (Bruckert et al., 2005). Still, the biochemical mechanisms underlying these common reactions are effectively unknown.

Cholesterol is only one in a whole series of endogenous metabolites which are dependent on the synthesis of mevalonic acid (Grunler et al., 1994), among them dolichol, ubiquinone, protein isoprenyl anchors, and isopentenyl pyrophosphate, which is required for the posttranscriptional maturation of selenocysteine-tRNA, an essential component of selenoprotein synthesis (Warner et al., 2000; Hatfield et al., 2006). By virtue of their inhibition of HMG-CoA-reductase, statins may therefore clearly influence a variety of other cellular functions beyond cholesterol homeostasis, both beneficially and adversely. This concept has usually been termed the pleiotropy of statin action (Corsini et al., 1999; Ray et al., 2006; Wang et al., 2008).

The biochemical possibility that statins might be modulators of selenoprotein expression has recently been discussed pertaining to a variety of clinical and pharmacological issues, particularly their untoward side-effects (Moosmann and Behl, 2004a; Moosmann and Behl, 2004b; Noël, 2004; Hoffmann and Berry, 2005; Rederstorff et al., 2006; Suzuki et al., 2008). The main clinical evidence that such a modulation might indeed occur in humans has been the observation that the pathological presentation of statin-induced myopathy was strikingly similar to myopathic conditions evoked by nutritional selenium deficiency or functional mutations in selenoprotein N (Moosmann and Behl, 2004a). These diagnostic findings could be rationalized on the basis of earlier biochemical investigations showing that selenocysteine-tRNA maturation and selenoprotein synthesis in ovarian and breast carcinoma cells were in fact dependent on metabolites of the mevalonate pathway (Diamond et al., 1996; Warner et al., 2000). Nevertheless, a systematic experimental investigation into the biochemical and functional consequences of statin treatment on selenoprotein expression in human cells has not been reported yet.

In the following, we have investigated the effects of varying concentrations of atorvastatin, cerivastatin, and lovastatin on selenoprotein synthesis in human HepG2 hepatocytes (Javitt,

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1990). We have found that clinically attained levels of these drugs lead to a significant loss of glutathione peroxidase expression and catalytic activity, resulting in an increased vulnerability of the cells to secondary toxins.

## Materials and Methods

**Materials.** Media and cell culture reagents were purchased from Invitrogen. HepG2 cells were a kind gift from Dr. Alain Lescure, Strasbourg. Atorvastatin and cerivastatin were obtained from Synfine, lovastatin was from Sigma. Radioactive  $^{75}\text{Se}$  ( $\text{H}_2\text{SeO}_3$ ;  $6 \times 10^{13}$  Bq/g Se) was from the University of Missouri Research Reactor Center (MURR). The BCA Protein Assay Kit was obtained from Pierce. Rabbit anti-GPx4 and mouse anti-tubulin antibodies were purchased from Abcam; HRP-conjugated secondary antibodies were from Jackson ImmunoResearch. Immobilon Western chemiluminescent HRP substrates were from Millipore. The Absolutely RNA Miniprep Kit was from Stratagene, the Omniscript Reverse Transkription Kit was from Qiagen, and the Absolute SYBR Green Fluorescein Mix was from Thermo Scientific. PCR primers were synthesized by MWG Biotech. All other chemicals and biochemicals were obtained from Sigma if not otherwise indicated.

**Cell Culture.** HepG2 cells were maintained in a 5%  $\text{CO}_2$  humidified incubator at  $37^\circ\text{C}$ . Maintenance medium was DMEM containing FCS (10%), penicillin/streptomycin (100 U/ml), pyruvate (1 mM), Hepes (20 mM), non-essential amino acids (1% of a commercial stock solution from Invitrogen), and 25 nM sodium selenite ( $\text{Na}_2\text{SeO}_3$ ). Cell culture plates were coated with poly-L-ornithine (1 mg/ml) for at least 15 min and washed with PBS before being used for cell culture. All experiments were performed in serum-free Optimum medium containing penicillin/streptomycin (100 U/ml), Hepes (20 mM), and 25 nM sodium selenite unless otherwise stated. Statins were dissolved in ethanol; the control of each experiment (“untreated”) was incubated with an equal volume of this vehicle (0.1-1%).

**Western Blotting.** HepG2 cells were seeded on day 0 in coated 12-well plates at a density of  $10^5$  cells/well in 1 ml medium. On day 1, the cells were incubated with different concentrations of atorvastatin, cerivastatin, or lovastatin. After 4 d incubation, the cells were harvested in 200  $\mu\text{l}$  lysis buffer consisting of 0.1 M Tris-HCl, pH 8.0, and 1 x metal chelator-free protease inhibitor cocktail (Sigma). Protein determination was done by BCA Protein

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Assay according to the manufacturer's protocol. The samples were normalized for protein content, and 10 µg of each sample were electrophoresed by 10% SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5% non-fat dry milk in TBST for 30 min, the membranes were washed and incubated at 4°C overnight with rabbit anti-GPx4 antibody diluted 1:1000 in 1.5% non-fat dry milk in TBST containing 0.1% sodium azide. For detection, anti-rabbit HRP-conjugated secondary antibody was diluted 1:10000 in 1.5% non-fat dry milk in TBST and applied for 1 h at RT. Enhanced chemiluminescence (ECL) was performed with Immobilon Western HRP substrates according to the manufacturer's instructions. A mouse anti-tubulin antibody was used as loading control.

**Selenoprotein Labeling.** Cells were seeded and incubated as described above (see Western Blotting), including incubation with atorvastatin, cerivastatin, or lovastatin for 4 d. In the reversibility experiments, the cells were concomitantly incubated with the different metabolites of the mevalonate pathway for 4 d. Before the end of each experiment, the cells were incubated with 37 kBq/ml <sup>75</sup>Se for 16 h. A non-radioactive control experiment was carried out in parallel for protein determination. The <sup>75</sup>Se-labeled cells were harvested in reducing SDS lysis buffer and boiled for 15 min at 95°C. After adjustment for equal protein content, ~150 µg protein of each sample were electrophoresed (10% SDS-PAGE). Following staining with Coomassie blue, the gel was dried and exposed to a Phosphorimager plate (Fuji), which was read after 5 d of exposition. As a second quantification, the indicated GPx and TRxR regions of the stained and dried gels were cut out, solubilized in liquid scintillation cocktail (Zinsser Analytic) at 45°C overnight, and analyzed in a scintillation counter (Perkin-Elmer).

**GPx Activity.** HepG2 cells were seeded on day 0 in coated 6-well plates at a density of 2.5 x 10<sup>5</sup> cells/well in 2 ml medium. After an incubation of 4 d with atorvastatin or cerivastatin, the cells were harvested on day 5 in lysis buffer (0.1 M Tris-HCl, pH 8.0, 1 x protease inhibitor cocktail). For the preparation of a cytosolic fraction, the cells were sonicated and centrifuged



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at 105000 g for 1 h at 4°C. The supernatant was taken for protein determination (BCA Protein Assay Kit) and GPx activity measurements (Saito and Takahashi, 2002). To this end, aliquots of each sample were adjusted to 0.2 mM NADPH, 2 mM GSH, and 1 U glutathione reductase in 0.1 M Tris-HCl, pH 8.0, to reach a final volume of 1 ml. After preincubation of the assay mixture for 2 min at RT, the reaction was started by adding 70  $\mu$ mol *tert*-butyl hydroperoxide (tBuOOH). The oxidation of NADPH was recorded at 340 nm and transformed into U/mg protein. The value of the untreated control cells was set as 100%.

**Glutathione quantification.** Cells seeded on day 0 in 12-well plates were incubated with different concentrations of statins as described above (see Western Blotting). Total (GSH + GSSG) and oxidized (GSSG) glutathione were measured essentially as published (Griffith, 1980). In brief, cells harvested on day 5 in 4°C MES buffer (50 mM MES, 1 mM EDTA, pH 6.0) were shortly sonicated and centrifuged at 10000 g for 15 min at 4°C. Aliquots of the supernatants were de-proteinated by incubation with 1 vol of 10% metaphosphoric acid for 5 min at RT. Following centrifugation at 2000 g for 2 min, the supernatants were stored at -20°C. Prior to the photometric measurement, the de-proteinated samples were adjusted to pH 5.5 with 4 M triethanolamine, mixed with 4 vol of reagent solution (3 mM NADPH, 6 mM DTNB, both dissolved in 125 mM sodium phosphate, 6.3 mM EDTA, pH 7.5), and developed with 50 U/ml glutathione reductase. After 10 min incubation at 30°C, the samples were measured photometrically at 405 nm to determine total glutathione (GSH + GSSG). Oxidized glutathione (GSSG) was quantified by derivatizing the initially reduced glutathione (GSH) with 10 mM 2-vinylpyridine (60 min at RT), followed by the same procedure as described above. Glutathione contents were normalized to the cytosolic protein concentration, which was quantified by the BCA method.

**Reactive oxygen species production.** Intracellular ROS were quantified by the broad-spectrum fluorescent oxidant probe 2',7'-dichlorofluorescein (DCF) (Moosmann et al., 2001). To this end, HepG2 cells were seeded in 96-well plates at a density of  $8 \times 10^3$  cells/well in

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0.1 ml medium and treated with the indicated statin concentrations one day later. On day 5, the cells were incubated with 1  $\mu$ M cell-permeable DCF diacetate (DCFDA) and immediately transferred to a multiwell fluorescence plate reader (Wallac) heated to 37°C, to monitor the increase in fluorescence at 485 nm excitation / 535 nm emission. After 1 h, the cells were removed from the counter to normalize the obtained results to metabolic activity, which was determined by MTT assay as described as follows (Cytotoxicity Assays).

**Cytotoxicity Assays.** HepG2 cells were seeded on day 0 in coated 96-well plates at a density of  $8 \times 10^3$  cells/well in 0.1 ml serum-free medium supplemented with the indicated concentrations of sodium selenite. Statins were added to the cells on the next day. Following 3 d of incubation, the cells were challenged with varying concentrations of tBuOOH as denoted. After 24 h, cell viability was analyzed by incubation of the cells for 1.5-2 h with 10  $\mu$ l of an aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml). The generated formazan crystals were dissolved with 100  $\mu$ l solubilization solution (10% SDS, 40% dimethylformamide, pH 4 with acetic acid) as described (Moosmann et al., 2001). The resulting purple color was measured with a plate reader at 560 nm.

**Quantitative RT-PCR.** HepG2 cells were seeded and incubated as described above (see GPx Activity). On day 5, RNA was isolated with the Absolutely RNA Miniprep Kit. cDNA synthesis was carried out on 1  $\mu$ g RNA using the Omniscript Reverse Transkription Kit following the manufacturer's protocol. Real-time RT-PCR conditions were as follows: 30 s at 94°C, 30 s at 60°C, 60 s at 72°C; 35 cycles. The employed primers are shown in Table 1. Relative expression levels were calculated using a pairwise fixed reallocation randomization test.

**Statistics.** Statistically evaluated data represent mean  $\pm$  standard deviation of at least three independent experiments. The statistical significance of differences between groups was

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calculated by one-way ANOVA, followed by Student-Newman-Keul's test. Values of  $p < 0.05$  or  $p < 0.01$  were considered significant, as detailed in the corresponding figure legends.

## Results

The expression of selenoproteins in HepG2 cells upon treatment with atorvastatin, cerivastatin, or lovastatin was examined by  $^{75}\text{Se}$ -labeling of newly synthesized selenoproteins. The major selenoproteins expressed in HepG2 cells under standard culture conditions (containing 25 nM selenite; see Materials and Methods) were found to be thioredoxin reductase (TrxR) and glutathione peroxidase (GPx). All of the tested statins, atorvastatin (Fig. 1A) as well as cerivastatin (Fig. 1B) and lovastatin (Fig. 1C), strongly reduced the synthesis of the two predominant GPx isoforms, GPx1 (running at ~25 kDa) and GPx4 (running at ~20 kDa), whereas TrxR biosynthesis appeared to be unaffected. Cerivastatin was found to be more potent in the suppression of GPx biosynthesis than the other statins, which correlates with the differential cholesterol-lowering potential of these drugs (Chong et al., 2001).

To investigate whether the statin-induced decrease in the de novo synthesis of glutathione peroxidases also resulted in reduced steady-state levels of these enzymes, total GPx4 was analyzed by Western Blotting (Fig. 2). The treatment of HepG2 cells with nanomolar concentrations of atorvastatin (Fig. 2A), cerivastatin (Fig. 2B), or lovastatin (Fig. 2C) in fact resulted in a pronounced, concentration-dependent loss of GPx4 immunoreactivity.

The statin-induced stalling of GPx expression was furthermore found to result in a significant decrease of cytosolic, glutathione-dependent peroxidase activity. Using an NADPH-coupled enzyme assay to measure total cytosolic GPx activity towards *tert*-butyl hydroperoxide (tBuOOH), a significant impairment of the cellular peroxide detoxification capacity was seen with 10 nM cerivastatin or 100 nM atorvastatin (Fig. 3A). With higher doses of these statins, HepG2 cells lost approximately 50-70% of all glutathione or NADPH-dependent peroxidase activity. The reduction of GPx activity had still only limited impact on glutathione levels or redox state. A major change in the levels total glutathione (GSH + GSSG) was not observed unless at the highest concentration of cerivastatin tested (1000 nM; Fig. 3B). At this concentration, the cells yet began featuring microscopic signs of toxicity (data not shown). A

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similar picture emerged when reactive oxygen species (ROS) accumulation in statin-treated cells was analyzed (Fig. 3C). Using reductive metabolic activity as normalization factor, a statistically significant increase in steady-state ROS levels was found only with the highest cerivastatin concentration, in spite of a non-significant trend towards higher ROS levels that was already seen at lower concentrations of both atorvastatin and cerivastatin.

In order to analyze the potential functional consequences of the observed loss of GPx activity, we have performed cytotoxicity experiments (Fig. 4) using an exogenously added peroxide as toxin (tBuOOH). As evidenced by Figure 3, this compound can be detoxified by glutathione peroxidase. Pretreatment of the cells with a fixed concentration of atorvastatin (1  $\mu$ M) or cerivastatin (100 nM) resulted in a drastically decreased ability to survive any following tBuOOH challenge (Fig. 4A). Under the conditions of this experiment, the employed statin concentrations were not or only marginally toxic by themselves (less than 15% reduction of cell viability; data not shown). To investigate whether the statin-induced vulnerability increase was potentially attributable to selenoprotein downregulation, reversibility experiments with elevated levels of added selenium were performed. In the absence of statins, selenium supplementation (0, 25, 250 nM) did not modulate baseline cell viability or peroxide resistance over a wide range of peroxide concentrations (Fig. 4B), in spite of the fact that GPx expression strongly correlated with the availability of this trace element (Fig. 4E). In fact, the cells were almost fully resistant to tBuOOH up to the highest tested concentration of 550  $\mu$ M (Fig. 4B), irrespective of the added selenium concentration. In statin-treated cultures, however, selenium supplementation suddenly became a decisive factor to result in a significantly increased capacity of the cells to survive the peroxide challenge (Fig. 4B,C). These results support the idea that selenoprotein suppression is causally involved in statin-induced hepatocyte impairment.

The molecular origin of the observed suppression of GPx synthesis by statins was analyzed by RT-PCR. At identical concentrations as in the above experiments, neither atorvastatin (1  $\mu$ M),

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nor cerivastatin (100 nM) or lovastatin (1  $\mu$ M) led a measureable decrease in GPx1 or GPx4 transcript levels, whereas HMG-CoA-reductase was unanimously induced as expected (Fig. 5A-C). In addition, a partially significant induction of two genes (heme oxygenase 1 (HO1) and NAD(P)H:quinone oxidoreductase 1 (NQO)) regulated by the oxidative/electrophilic stress-induced Nrf2 detoxification pathway was observed (Fig. 5D). This pathway has been shown to be induced in liver as secondary response to selenoprotein deficiency (Suzuki et al., 2008). These findings seem to rule out transcriptional effects as cause of the statin-induced effects on selenoprotein synthesis.

In order to further track down the apparently posttranscriptional mechanism of statin-induced selenoprotein modulation, metabolic reconstitution experiments were performed. Statin-treated cells were incubated with high, but still non-toxic concentrations of selected intermediates of the mevalonate pathway. The results in Figure 6 show that only mevalonic acid was capable of fully restoring selenoprotein synthesis. A partial rescue was seen with geranylgeraniol. Notably, isoprenes required for the posttranslational modification of small heterotrimeric G proteins, such as farnesyl or geranylgeranyl pyrophosphate, were inefficient in restoring selenoprotein synthesis, in spite of their functionally established cell permeability and activity at the employed concentrations (Hirai et al., 1997; Woo et al., 2005; Liang et al., 2006). These data are consistent with all molecular mechanisms dependent on isopentenyl pyrophosphate, such as selenocysteine-tRNA maturation, but essentially rule out mechanisms resting solely upon the long-chain isoprenylation of proteins or cofactor precursors. The single effect of geranylgeraniol requires further investigation.

## Discussion

Cholesterol biosynthesis is only one of the functions of the mevalonate pathway. Except for providing an endogenous source of steroids, it is essential for the synthesis of diverse isoprenoids such as dolichol or farnesyl pyrophosphate (Grunler et al., 1994). The exceptional importance of this pathway has been reasserted by the discovery of mevalonate kinase deficiency, a congenital disorder caused by deficiency of an early enzyme of the mevalonate pathway acting directly downstream of HMG-CoA reductase (Hoffmann et al., 1986). The affected patients show severe pathological abnormalities in multiple organs, whereas their cholesterol levels are normal, which may be explained by the fact that cholesterol can be sufficiently contributed by the diet (Hoffmann et al., 1993). Hence, this disease may be viewed as a prime example for the pivotal importance of the non-sterol products of the mevalonate pathway, and likewise as a model for the consequences to be expected from a complete blockade of HMG-CoA reductase.

We have investigated the effect of HMG-CoA reductase inhibitors on one of the essential branches of the mevalonate pathway in human HepG2 hepatocytes, i.e. the biosynthesis of selenoproteins. Selenoprotein expression in mammals is dependent on a single tRNA species, selenocysteine-tRNA, which occurs in several variants distinguished by differential posttranscriptional modification (Hatfield et al., 2006). One of the most notable modifications is the isopentenylation of adenosine 37, which is dependent on the intracellular availability of isopentenyl pyrophosphate (Diamond et al., 1996; Warner et al., 2000). This modification has been characterized in different transgenic mouse models, in which it was found to be essential for the translation of selected, but not all selenoproteins in a highly tissue-specific manner (Moustafa et al., 2001). In general, those selenoproteins were seen to be preferentially modulated by a lack of selenocysteine-tRNA isopentenylation that would also respond most notably to declining selenium concentrations.

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Treating HepG2 cells with three different statins, we have found that all compounds possessed a pronounced potential to reduce the synthesis (Fig. 1), steady-state level (Fig. 2), and enzymatic activity (Fig. 3) of glutathione peroxidase, whereas the expression of thioredoxin reductase was unaffected. This finding corresponds with our observation that in selenium-depleted cell culture medium, HepG2 cells rapidly lose GPx, but not TrxR (data not shown). The concentrations needed to elicit significant GPx suppression were found to be approximately 100 nM for atorvastatin, 10 nM for cerivastatin, and 100 nM for lovastatin. These numbers are in full accordance with clinically attained drug concentrations in human plasma after single-dose administration, which have been reported to reach 50-120 nM for atorvastatin, 5 nM for cerivastatin, and 25-50 nM for lovastatin ( $C_{\max}$  following 40 mg atorvastatin, 0.2 mg cerivastatin, or 40 mg lovastatin orally) (Corsini et al., 1999). Moreover, HepG2 cells are known to express basal levels of cytochrome P450 isoforms CYP3A4 and CYP2C8 (Westerink and Schoonen, 2007), by which these statins are primarily degraded (Corsini et al., 1999). Hence, it is plausible to assume that the average concentrations in the cell culture medium over the 4-day incubation period used in most experiments were even lower than the above-cited concentrations of 10 and 100 nM, which were applied to the cells only once at the beginning of each experiment.

Selenoprotein expression is well-known to be essential for the maintenance of liver function, at least in rodents (Carlson et al., 2004). Moreover, selenium appears to be a protective factor against the development of hepatocellular carcinoma in high-risk patients (Yu and Yuan, 2004). Interestingly, transgenic mice lacking selenoprotein expression exclusively in hepatocytes were phenotypically normal until shortly before their premature death from acute liver failure, which occurred variably between 3 and 24 weeks of age (Carlson et al., 2004). Hence, the liver seems to be capable of compensating a loss of selenoprotein expression to a significant degree, but not indefinitely. This conclusion is supported by the observation that the oxidative/electrophilic stress-induced Nrf2 detoxification pathway was found to be



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strikingly activated in response to liver-specific selenoprotein deficiency (Suzuki et al., 2008), a finding that was essentially recapitulated in this work (Fig. 5D), in which yet only GPx expression was reduced instead of global selenoprotein expression. Moreover, targeted disruption of Nrf2 in the selenoprotein-deficient mouse liver led to dramatically accelerated hepatocellular degeneration and mortality (Suzuki et al., 2008). These results correlate well with our observation that common concentrations of statins and the resulting GPx suppression are not generally cytotoxic by themselves, whereas they do entail a reduction in cellular detoxification capacity (Fig. 3,4). We assume that a diminished capability of the statin-treated liver to withstand occasionally arising peak concentrations of oxidative toxins of either endogenous or exogenous origin may explain the clinically observed elevations in liver enzymes as well as their elusive patterns of occurrence (Jacobson, 2006).

The idea of a decreased antioxidative detoxification capacity of the liver upon statin treatment is backed by a number of characteristic *in vivo* findings. For instance, lovastatin administration to rats has been described to result in a significantly increased liver membrane peroxidizability (Lankin et al., 2007), as would be indicative of a loss of GPx4. Moreover, LDL from statin-treated patients has been shown in several studies to possess decreased antioxidative capacity if properly isolated (Palomaki et al., 1999; Lankin et al., 2003). Still, it is important to note that the latter parameters reflect specific aspects of intracellular antioxidative capacity (the LDL precursor is assembled intracellularly); hence, their prooxidative response to statin treatment is not contradictory to reports describing antioxidative effects of statins regarding certain plasma/endothelial markers of oxidative stress (Rosenson, 2004). These may be largely dependent on the inflammatory activity in the endothelium, which is clearly reduced by all statins (Moosmann and Behl, 2004b; Rosenson, 2004). In addition, it is well possible that some of the compensatory enzymes induced by Nrf2 may actually overcompensate the primary prooxidative effect of these compounds with respect to different markers (Suzuki et al., 2008). Thus, the selenoprotein-modulatory effect

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of the statins may not be directly reflected in global markers of redox homeostasis such as glutathione (Fig. 3B), or vascular indices of oxidative stress such as NADPH-dependent superoxide production, but it may nevertheless become visible under special conditions of tolerance testing.

In summary, our results demonstrate that two apparently disparate metabolic pathways, cholesterol biosynthesis and selenoprotein synthesis, are functionally interconnected, which provides a plausible and explanatory origin for the hepatic side-effects of statin treatment in humans.

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## Acknowledgements

We are indebted to A. Lescure, K. Kivistö, A. Weichert, J. Mocko, H.-J. Breter, G. Gimpl, and C. Behl for their ample advice and generous support.

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## Footnotes

This work was supported by the Peter-und-Beate-Heller-Stiftung and the Forschungsfonds der Universität Mainz.

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## Figure Legends

### Figure 1. <sup>75</sup>Se-labeling of selenoproteins in statin-treated HepG2 cells.

HepG2 cells were incubated with different concentrations of (A) atorvastatin, (B) cerivastatin, or (C) lovastatin for 4 d, including 16 h incubation with 37 kBq/ml <sup>75</sup>Se. Labeled proteins were separated by SDS-PAGE and quantified by Phosphorimager (left panels) and scintillation counting (tables). The dried, protein-stained gels (loading control) are shown in the central panels.

### Figure 2. Cellular GPx4 expression after statin treatment.

Cells were incubated for 4 d with different concentrations of (A) atorvastatin, (B) cerivastatin, or (C) lovastatin as indicated. GPx4 expression levels were quantified by Western Blotting using commercial antibodies. Equal amounts of protein according to BCA assay were blotted; tubulin was used as a loading control. The panels on the right show quantifications (mean  $\pm$  SD) of three independent blots as depicted on the left. \* $p$ <0.05 compared to the control.

### Figure 3. Effect of statins on glutathione peroxidase activity, glutathione levels, and ROS production.

HepG2 cells were incubated with atorvastatin (left) or cerivastatin (right) for 4 d. (A) Glutathione peroxidase activity in the cytosolic compartment was measured by means of a coupled enzyme assay using tBuOOH as substrate. The results were normalized for protein content and represent mean  $\pm$  SD of three independent determinations; the activity of untreated control cells was set as 100%. \* $p$ <0.05 compared to the control. (B) Total glutathione content (GSH + GSSG) and oxidized glutathione (GSSG) were determined by cyclic DTNB reduction assay with or without vinylpyridine derivatization. Results normalized for protein content are shown as mean  $\pm$  SD of three independent experiments.

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Total glutathione levels of untreated control cells were set as 100%.  $*p < 0.05$  compared to the control. (C) ROS production was measured by fluorescent DCFA assay. Reductive metabolic activity was used for normalization. The depicted data represent one of three independent experiments, each performed in quadruplicates. Relative ROS production (mean  $\pm$  SD) in response to statin treatment was calculated by setting statin-free cultures as 100%.  $*p < 0.05$  compared to vehicle-treated control cells.

## Figure 4. Viability of statin-treated HepG2 cells after peroxide administration.

MTT tests were performed to investigate the effects of atorvastatin (1  $\mu$ M) and cerivastatin (100 nM) on the susceptibility of HepG2 cells towards *tert*-butyl hydroperoxide (tBuOOH) toxicity. (A) Cells preincubated with atorvastatin or cerivastatin for 3 d were challenged with increasing concentrations of tBuOOH in medium without added selenium.  $*p < 0.01$  compared to statin-untreated cells. (B-D) Cells in medium containing different concentrations of added selenium (0, 25, 250 nM) were either (B) left untreated, (C) incubated with atorvastatin, or (D) incubated with cerivastatin.  $*p < 0.01$  compared to selenium-unsupplemented cells. The depicted data represent one of three independent experiments, each performed in triplicates. Relative cell viabilities (mean  $\pm$  SD) after incubation with tBuOOH were calculated by setting the toxin-free cultures as 100%. (E) Western Blot analysis of GPx4 expression in cells treated with atorvastatin (1  $\mu$ M) or cerivastatin (100 nM) and different concentrations of added selenium (0, 25, 250 nM) for 4 d. Tubulin immunoreactivity is shown as loading control.

## Figure 5. Quantitative RT-PCR analysis of GPx4, GPx1, HMGCR, HO1, and NQO mRNA levels.

(A-C) Cells were incubated with (A) 1  $\mu$ M atorvastatin, (B) 100 nM cerivastatin, or (C) 1  $\mu$ M lovastatin for 4 d, after which total mRNA was isolated for quantitative analysis of GPx4, GPx1 and HMGCR transcription. (D) Relative transcript levels of HO1 and NQO in response

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to 4 d treatment with 1  $\mu$ M atorvastatin or 100 nM cerivastatin. Results for all genes were normalized to tubulin transcription before expression ratios compared to vehicle-treated control cells were calculated. Data are shown as mean  $\pm$  SD on a log<sub>2</sub>-based scale (n=4; n=2 for HMGCR). \* $p$ <0.05 compared to statin-untreated cells.

**Figure 6. Reversibility of statin-induced selenoprotein modulation by metabolites of the mevalonic acid pathway.**

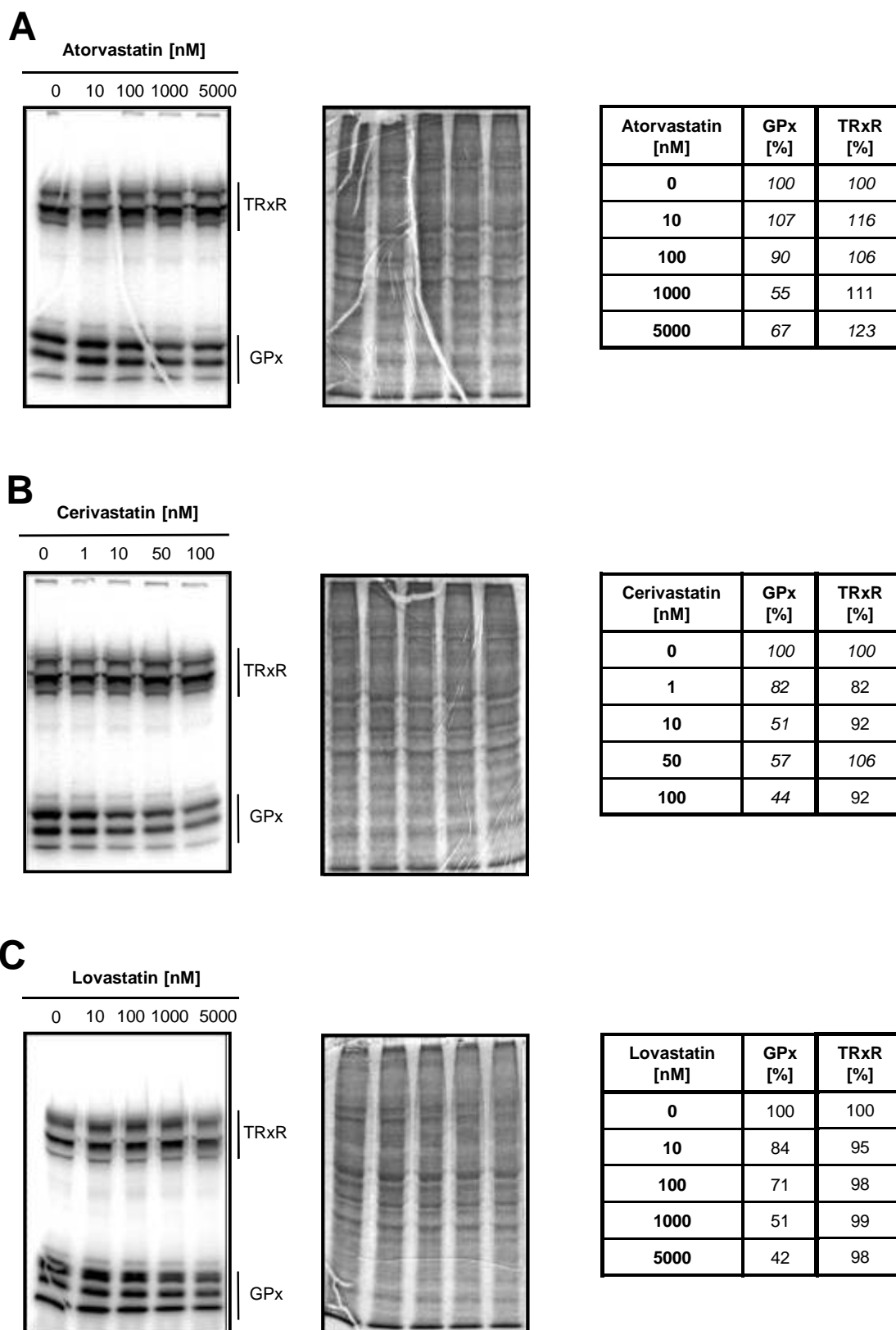
HepG2 cells were incubated with 500 nM cerivastatin and 10  $\mu$ M of different metabolites of the mevalonic acid pathway (mevalonolactone 1 mM) for 4 d. Before harvesting, the cells were incubated with 37 kBq/ml <sup>75</sup>Se for 16 h. Samples were analyzed as described in Fig. 1. The abbreviations denote: Mev, mevalonolactone; G, geraniol; GPP, geranyl pyrophosphate; F, farnesol; FPP, farnesyl pyrophosphate; GG, geranylgeraniol; GPP, geranylgeranyl pyrophosphate.

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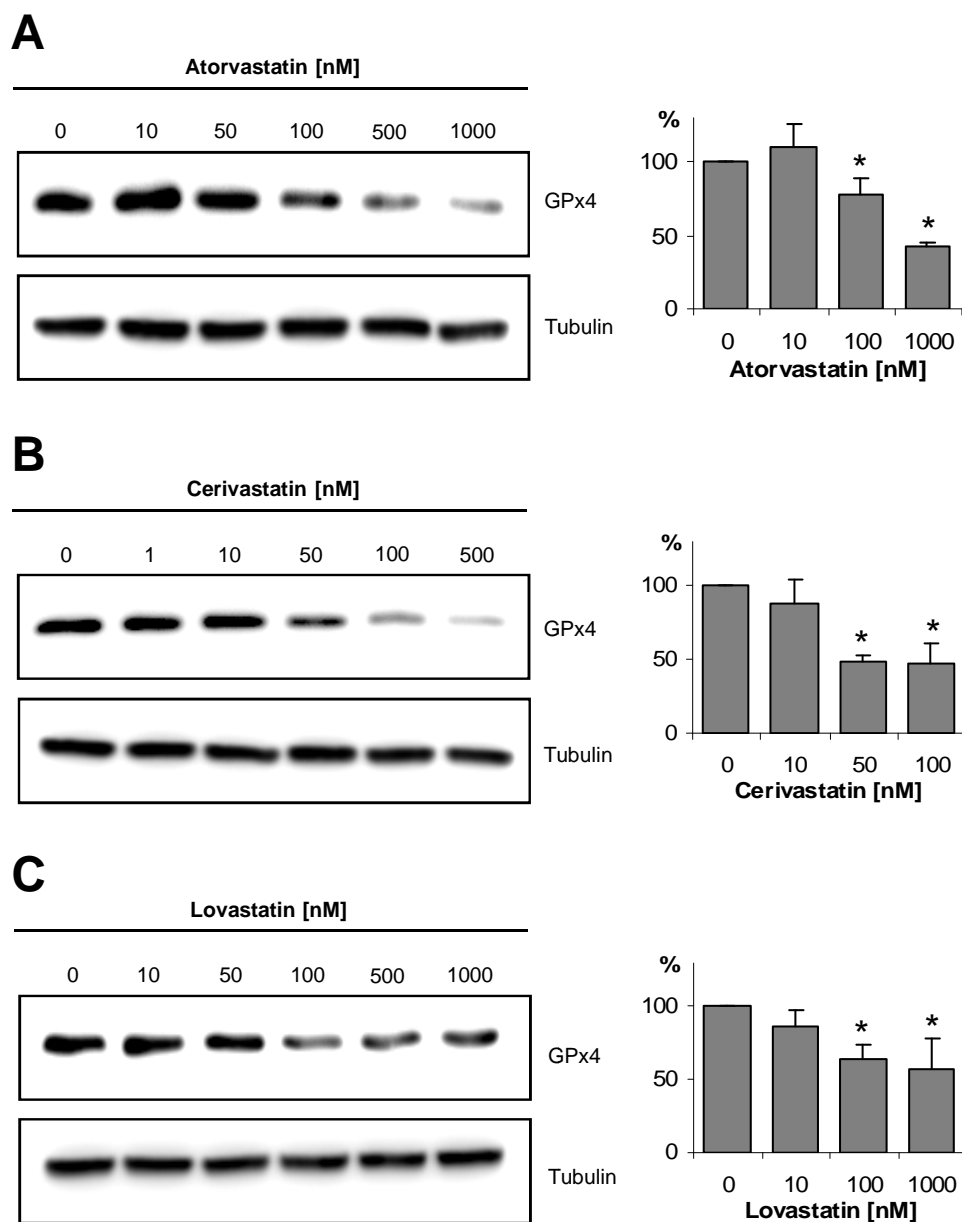
## Tables

**Table 1:** Primers used for real-time RT-PCR.

Primer name	Sequence 5' → 3'	Product size (bp)
GPx1 forward	GCA CCC TCT CTT CGC CTT C	207
GPx1 reverse	TCA GGC TCG ATG TCA ATG GTC	
GPx4 forward	CGG GCT ACA ACG TCA AAT TCG	222
GPx4 reverse	GGG GCA GGT CCT TCT CTA TCA	
HMGCR forward	GGA CCC CTT TGC TTA GAT GAA	107
HMGCR reverse	CCA CCA AGA CCT ATT GCT CTG	
HO-1 forward	CAG TGC CAC CAA GTT CAA GC	112
HO-1 reverse	GTT GAG CAG GAA CGC AGT CTT	
NQO forward	GGT TTG AGC GAG TGT TCA TAG G	129
NQO reverse	GCA GAG AGT ACA TGG AGC CAC	
Tubulin forward	CTG TTC GCT CAG GTC CTT TTG	147
Tubulin reverse	CCT CCT TCC GTA CCA CAT CCA	

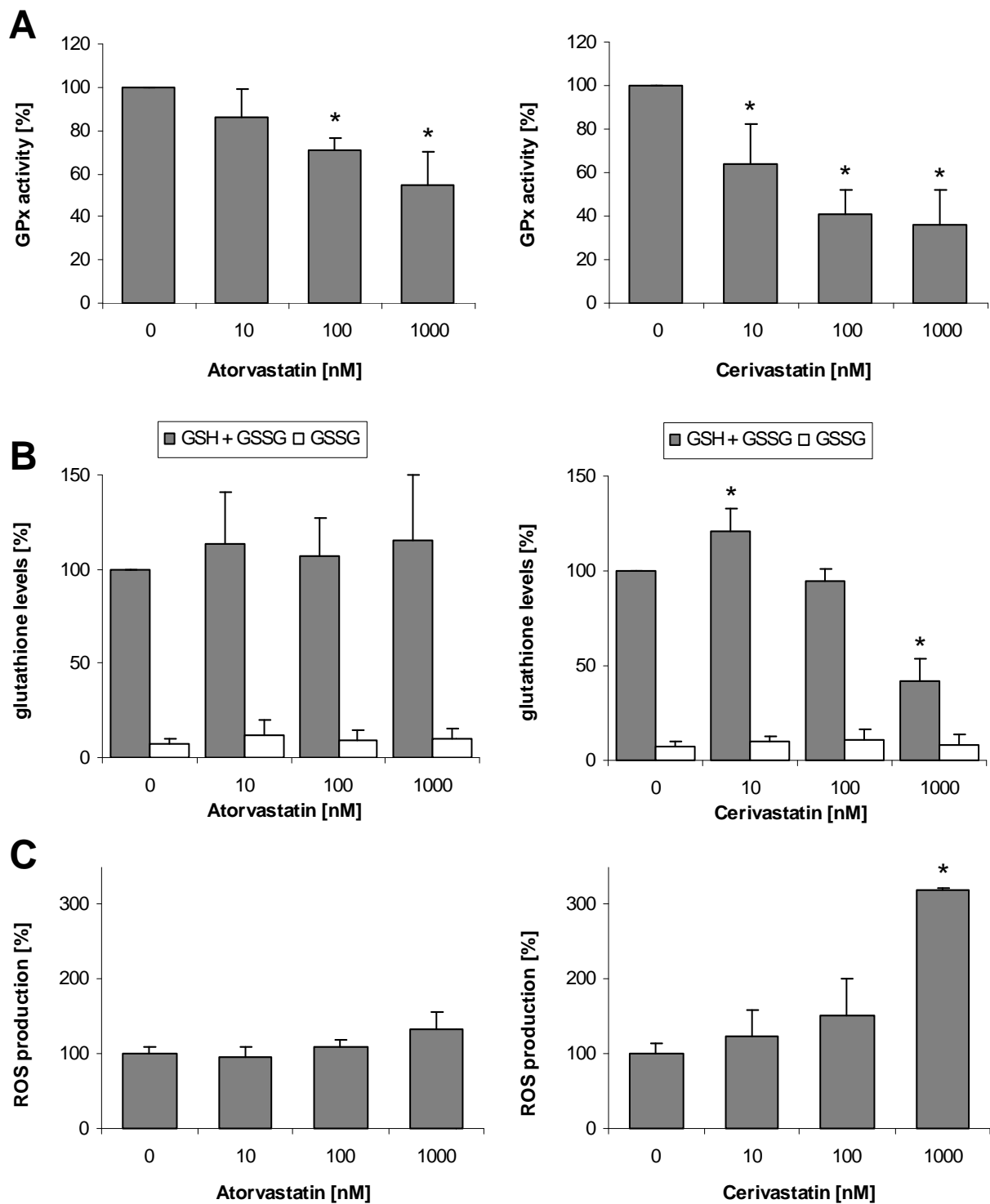


**Figure 1**

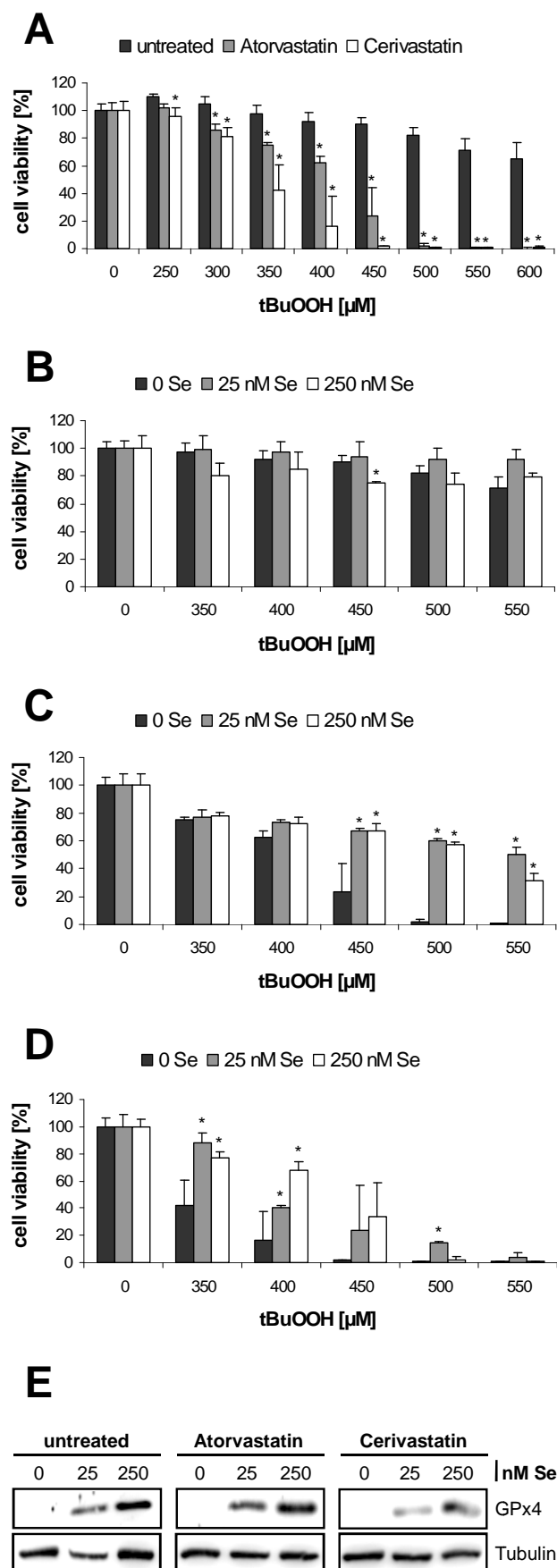


**Figure 2**

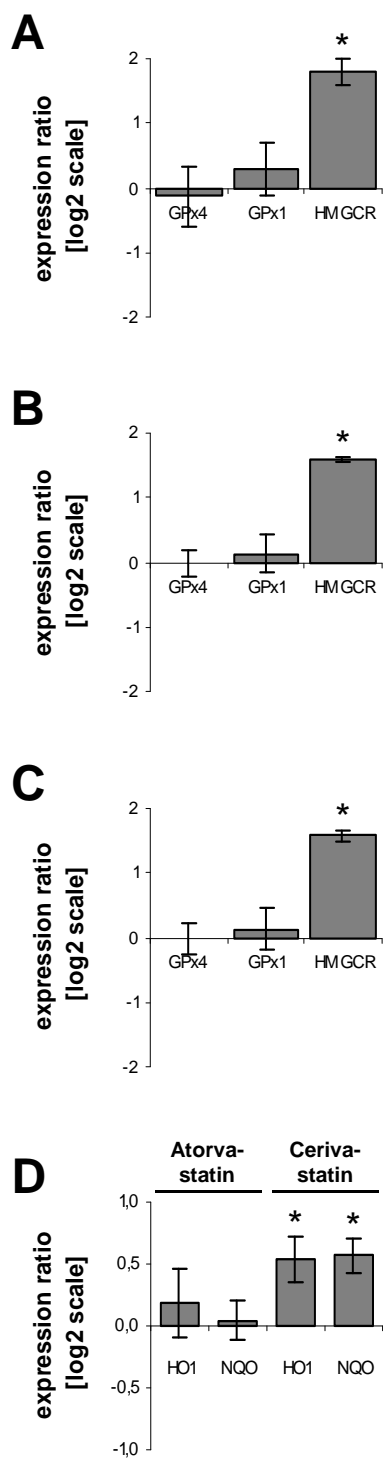




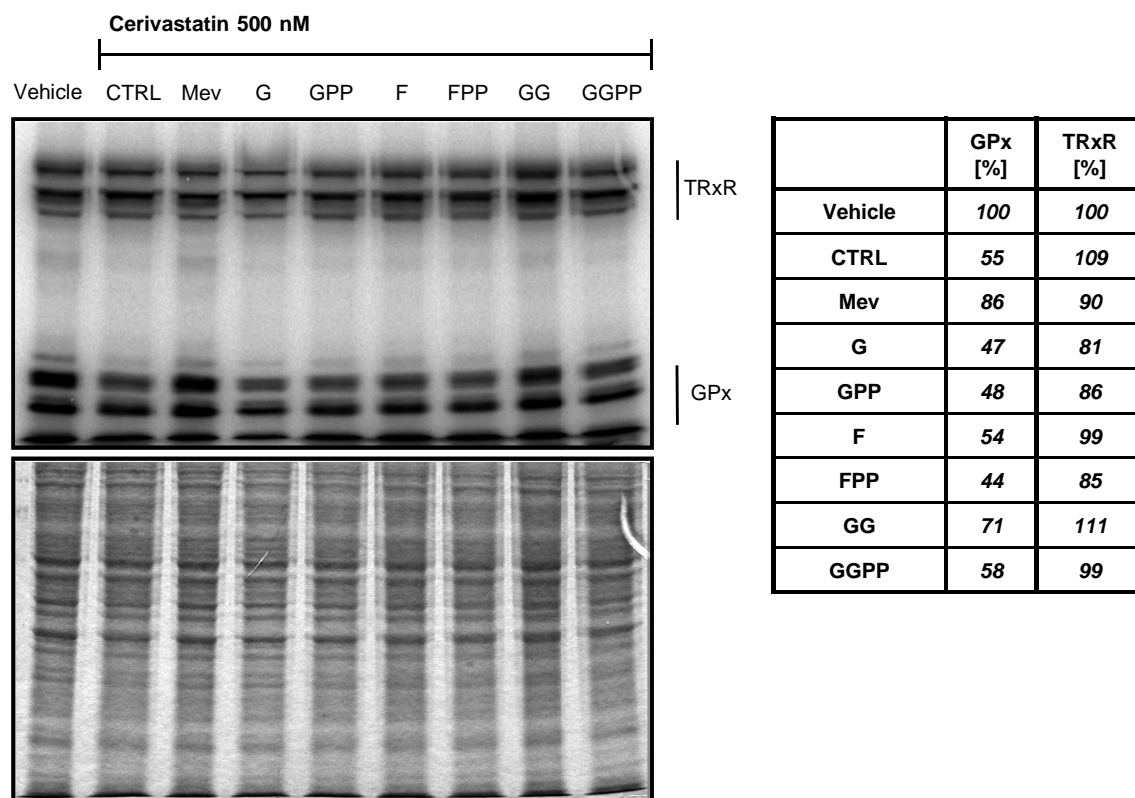
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**