Journal of Cellular and Molecular Medicine
A JOURNAL OF TRANSLATIONAL MEDICINE

J. Cell. Mol. Med. Vol 16, No 2, 2012 pp. 394-400

Atorvastatin activates heme oxygenase-1 at the stress response elements

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Received: December 28, 2010; Accepted: March 25, 2011

Abstract

Statins are known to inhibit growth of a number of cancer cells, but their mechanism of action is not well established. In this study, human prostate adenocarcinoma PC-3 and breast adenocarcinoma MCF-7 cell lines were used as models to investigate the mechanism of action of atorvastatin, one of the statins. Atorvastatin was found to induce apoptosis in PC-3 cells at a concentration of 1 μM, and in MCF-7 cells at 50 μM. Initial survey of possible pathway using various pathway-specific luciferase reporter assays showed that atorvastatin-activated antioxidant response element (ARE), suggesting oxidative stress pathway may play a role in atorvastatin-induced apoptosis in both cell lines. Among the antioxidant response genes, heme oxygenase-1 (HO-1) was significantly up-regulated by atorvastatin. Pre-incubation of the cells with geranylgeranyl pyrophosphate blocked atorvastatin-induced apoptosis, but not up-regulation of HO-1, suggesting that atorvastatin-induced apoptosis is dependent on GTPase activity and up-regulation of HO-1 gene is not. Six ARE-like elements (designated StRE1 [stress response element] through StRE6) are present in the HO-1 promoter. Atorvastatin was able to activate all of the elements. Because these StRE sites are present in clusters in HO-1 promoter, up-regulation of HO-1 by atorvastatin may involve multiple StRE sites. The role of HO-1 in atorvastatin-induced apoptosis in PC-3 and MCF-7 remains to be studied.

Keywords: atorvastatin • statin • heme oxygenase-1 • antioxidant response element • apoptosis

Introduction

Atorvastatin belongs to a class of drugs known as statins, and is mainly used to lower serum cholesterol level by inhibiting the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate by HMG-CoA reductase, a rate-limiting step in the cholesterol biosynthesis pathway. However, this also prevents the synthesis of downstream products such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), which are needed for post-translational activation (prenylation) of small GTPases such as Ras and Rho. Failure in the prenylation of these GTPases results in their inability to interact with a wide spectrum of functionally different downstream mediators to initiate cytoplasmic signalling pathways and to regulate cell cycle progression [1]. Hence, statins have a pleiotropic effect of inhibiting growth of a number of cancer cells, but their mechanism of action is still not well established [2]. The problem lies in the diversities of lipophilicity of statins and cell types used in the studies.

A number of statins have been shown to induce cell growth arrest and apoptosis of prostate and breast cancer cells in *in vitro* as well as *in vivo* studies [3–8]. The prevention of prenylation of the small GTPase RhoA has been implicated for the action of statin [9]. However, the downstream mediators have not been well characterized. A number of candidates have been reported, these include p21 [10], E2F transcription factor 1 (E2F-1) [11], caspase-7 [12], caspases-3 and -9 [13], insulin-like growth factor 1 receptor [14] and inducible nitric oxide synthase [15]. Besides statin-induced apoptosis, simvastatin- (at higher dose, 60 μM) induced necrosis mediated by calcineurin and mitochondrial dysfunction [16]. More recently, atorvastatin has been shown to induce cell death in PC-3 cells *via* autophagy [17].

In this study, we used prostate cancer PC-3 and breast cancer MCF-7 cells as models to investigate the mechanism of action of atorvastatin. We herein report that atorvastatin up-regulates heme oxygenase-1 (HO-1) in PC-3 and MCF-7 cells.

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doi: 10.1111/j.1582-4934.2011.01324.x

Materials and methods

Reagents

Atorvastatin calcium was purchased from AK Scientific, Inc. (Mountain View, CA, USA). GGPP and N-acetyl cysteine were products of Sigma-Aldrich (St. Louis, MO, USA). Zinc protoporphyrin (ZnPP) was purchased through EMD Chemicals, Inc. (Gibbstown, NJ, USA). Antibodies against human β-actin and HO-1 were purchased from Cell Signaling Technology (Danvers, MA, USA) and Enzo Life Sciences (Plymouth Meeting, PA, USA), respectively.

Cell lines and cell culture

Human prostate adenocarcinoma PC-3 and breast adenocarcinoma MCF-7 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained as monolayer cultures in Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B (complete medium) and were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Construction of reporter plasmids

The enhancer-luciferase reporter plasmids were constructed by inserting sequences of various response elements into the filled-in *Nhe*I/*Bgl*II sites of pGL3-promoter vector (Promega, Madison, WI, USA) or *Eco*RV site of pGL4-promoter vector (see below) *via* blunt-end ligation. These enhancer sequences were synthesized chemically as double-stranded oligomers with the following sequences (only the sense strands were shown): antioxidant response element (ARE) site of human NAD(P)H dehydrogenase, quinone 1 (NQO1) promoter [18], 5′-AAATCGCAGTCACAGTGACTCAGCAGAATC-3′; potential heat-shock response element/nuclear factor κB (HSE)/(NF-κB) binding site of p53 promoter [19], 5′-GGGGTTG ATGGGATTGGGGTTTTCCCCTCC-3′; forkhead box class 'O' transcription factor (FOXO) site of rat Bim promoter [20], 5′-ACTAGGGTAAACACGCCGGG-3′; E2F-1 consensus binding site, 5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′; stress response element (StRE) sites of human HO-1 promoter [21]: StRE1, 5′-CTTGGGAATGCTGAGTCGCGATTTC-3′; StRE2, 5′-TGCATTTCTGCTGCGTCATGTTTGG-3′; StRE3, 5′-CTAGATTTTGCTGAGTCACCAGTGC-3′; StRE5, 5′-TCTGTTTTCGCTGAGTCACGGTCCC-3′; HSE site of human HO-1 promoter [22], 5′-GCTTTCTGGAACCTTCTGGGACGCC-3′; sterol-regulatory element binding protein (SREBP) site of human HO-1 promoter [23], 5′-CCGCCTGGCCCACGTGACCCGCCGAG-3′ and SP1 site of human HO-1 promoter [24], 5′-GGAAGGACCCCACCCCCAGCCAGCT-3′. Internal control plasmid, pGL4.74 [hRluc/TK], was purchased from Promega. pGL4-promoter vector was created by inserting a *Bgl*II/*Hind*III fragment containing Simian virus 40 (SV40)-promoter (from pGL3-promoter vector) into the same sites of pGL4.10[luc2] vector (Promega).

RT-PCR

Cells were grown in 25 cm^2 flasks and treated with 10 μM (for PC-3 cells) or 50 μM (MCF-7 cells) atorvastatin or equal amount of dimethyl sulphoxide (DMSO; vehicle) for 48 hrs. Total RNA was extracted using NucleoSpin Nucleic Acid Purification Kits (Clontech, Palo Alto, CA, USA). First-strand cDNA was synthesized from 5 μg of total RNA using ThermoScript (Invitrogen) in a volume of 20 μl. PCR was done for 30 cycles using 1 μl of the first-strand cDNA, 10 pmol of gene specific primers and 2.5 units of JumpStart Taq DNA polymerase (Sigma-Aldrich) in a volume of 50 μl.

Luciferase reporter assay

Luciferase reporter assays were carried out as described in our earlier study [25]. Briefly, cells in 24-well plates were co-transfected in triplicates with 250 ng of enhancer-luciferase reporter plasmid and 25 ng of pGL4.74 [hRluc/TK] internal control plasmid, using Lipofectamine 2000 (Invitrogen). Six hours after transfection, the medium was replaced with fresh one containing 10 μM atorvastatin or same amount of DMSO (vehicle). At 48 hrs after transfection, the growth medium was removed, and the cells were rinsed twice with phosphate buffered saline and were lysed by shaking for 15 min. at 25°C with 100 μl of Passive Lysis Reagent (Promega). Aliquots of 20 μl of the cell lysates were assayed for firefly and renilla luciferase activities using a 20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA) and Dual-Luciferase Reporter Assay System (Promega). The results were expressed as relative luciferase activity (a ratio of the activities of firefly luciferase/renilla luciferase). Each experiment was repeated at least twice.

Measurement of cell survival

Cells were seeded in triplicates in 48-well plate in complete medium. At about 25% confluency, cells were treated with atorvastatin or vehicle (DMSO) for 48 hrs. To study the protective effects of various agents, the cells were pre-treated with the agents for 2 hrs prior to the addition of atorvastatin. Cell survival was determined using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) according to the protocol provided by the manufacturer. The colour developed was measured at 490 nm. Each experiment was repeated twice.

Western blot analysis

Cells treated with 10 μM (for PC-3 cells) or 50 μM (for MCF-7 cells) atorvastatin or vehicle for 48 hrs were harvested, lysed with 1× Laemmli sample buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich), sonicated for 2 × 15 sec. and centrifuged at 10,000 rpm for 15 min. at 4°C. Aliquots of 100 μg protein extract were analysed on 10% SDS-PAGE, and transferred to PVDF membranes. The blots were analysed by Western blot according to the procedure provided by WesternDot 625 kit (Invitrogen). Briefly, the blots were incubated in 8 ml of blocking buffer in a small plastic dish for 1 hr at room temperature with gentle agitation. Then they were incubated with the diluted primary antibody (1:1000 dilution) at 4°C overnight. After washing three times with 50 ml of 1× wash buffer, 5 min. each, blots were incubated with 8 ml of biotin-XX-goat anti-rabbit antibody (1:2000 dilution) in blocking buffer for 1 hr. They were washed three times with 50 ml of 1× wash buffer for 5 min. each, and then incubated with 8 ml of Qdot 625 streptavidin conjugate (1:2000 dilution) in blocking buffer for 60 min. at room temperature. Finally, the blots were washed three times with 50 ml of 1× wash buffer for 5 min. each, and once with 20 ml of ultra-pure water. The wet blots were placed on a UV trans-illuminator and pictures were taken with a Polaroid camera and orange filter.

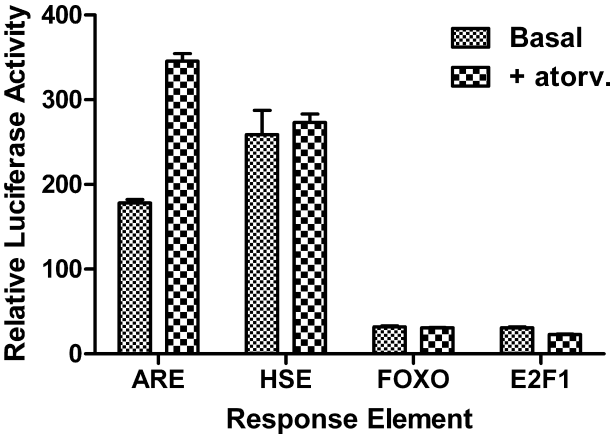

Fig. 1 Effect of atorvastatin on activities of ARE, HSE, FOXO and E2F1 elements in PC-3 cells. PC-3 cells were transfected with enhancer-luciferase reporter plasmid harbouring ARE site of human NQO1 promoter, potential HSE/NF-κB binding site of p53 promoter, FOXO site of rat Bim promoter or E2F-1 consensus binding site and pGL4.74 [hRluc/TK] internal control plasmid, treated with 10 μM atorvastatin for 48 hrs and luciferase activities were determined as described in 'Materials and methods'. Results were expressed as relative luciferase activity (mean ± S.D.).

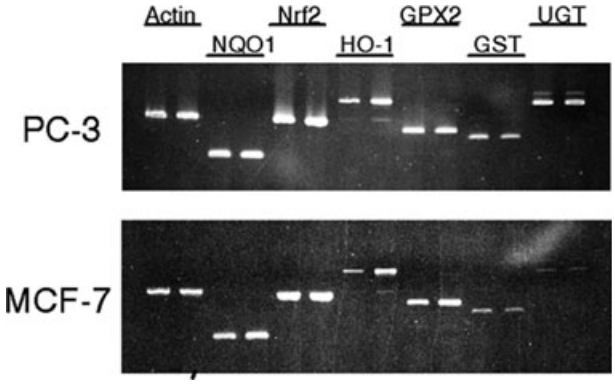

Fig. 2 Effect of atorvastatin on expression of various genes in PC-3 and MCF-7 cells. (Top) Total RNA was isolated from PC-3 cells treated with 10 μM atorvastatin or equal amount of DMSO (vehicle control) for 48 hrs, and expression of β-actin, NQO1, Nrf2, HO-1, GPX2, GSTM1 and UGT1A1 was analysed by RT-PCR using gene-specific primers. The PCR products were analysed on 2% agarose gel in pairs (left lane, control; right lane, atorvastatin-treated). (Bottom) Similar analysis by RT-PCR using RNA isolated from MCF-7 cells treated with 50 μM atorvastain or vehicle for 48 hrs.

Data analysis

Data points shown represent mean ± S.E. Statistically significant differences between data points of two groups were determined by Student's t-test. By convention, a *P* value of <0.05 was considered statistically significant.

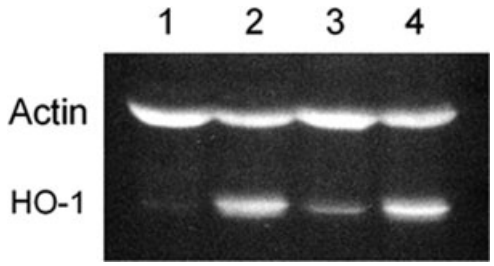

Fig. 3 Western blot analysis of HO-1 protein induced by atorvastatin in PC-3 and MCF-7 cells. PC-3 and MCF-7 cells were treated with 10 and 50 μM atorvastatin, respectively, for 48 hrs. Total proteins (100 μg) were analysed by Western blot as described in 'Materials and methods'. Samples are: (1) MCF-7 vehicle control, (2) MCF-7 treated with atorvastatin, (3) PC-3 vehicle control and (4) PC-3 treated with atorvastatin. Immunoreactive protein bands detected by WesternDot 625 appeared as fluorescent bands.

Results

Atorvastatin induced significant cell death in PC-3 cells at a concentration of 1 μM (data not shown). However, 10 μM gave the highest cell death. All subsequent experiments using PC-3 cells were done with 10 μM atorvastatin. MCF-7 cells were more resistant to atorvastatin; significant cell death was observed only when atorvastatin concentration reached to 50 μM (data not shown), although significant cell death at 10 μM was reported [8]. To investigate the mechanism of action of atorvastatin, luciferase-reporter constructs available in the laboratory were used to test the induction of gene expression by atorvastatin. When PC-3 cells were transfected with luciferase-reporter constructs containing ARE site of NQO1 promoter, potential HSE/NF-κB site of p53 promoter, FOXO site of Bim promoter or E2F-1 consensus binding site and induced with atorvastatin for 48 hrs, 2-fold induction of ARE site was observed (Fig. 1). Atorvastatin had no effect on HSE/NF-κB, FOXO and E2F-1 binding sites. This suggests that atorvastatin may be involved in the Keap1/Nrf2 (nuclear factor [erythroid-derived 2]-like 2) signalling pathway. To investigate which of the antioxidant response genes was induced by atorvastatin, total RNA was isolated from PC-3 and MCF-7 cells treated with atorvastatin, and expression of NQO1, Nrf2, HO-1, glutathione peroxidase 2 (GPX2), glutathione S-transferase μ_1 (GSTM1) and UDP glucuronosyltransferase 1, polypeptide A1 (UGT1A1) was evaluated by RT-PCR using gene-specific primers. Only HO-1 was found significantly induced (Fig. 2). There was also a slight increase in the expression of NQO1 and GXP2 in MCF-7 cells. The failure in detecting an increase of expression of these genes in PC-3 cells may be due to their relatively high basal expression. Minor bands of lower molecular weight HO-1 and higher molecular weight of UGT were also observed in Figure 2, these may represent unspecific PCR products or alternatively spliced mRNA. Induction of HO-1 by atorvastatin in both PC-3 and MCF-7 cells was confirmed by Western blot analysis (Fig. 3).

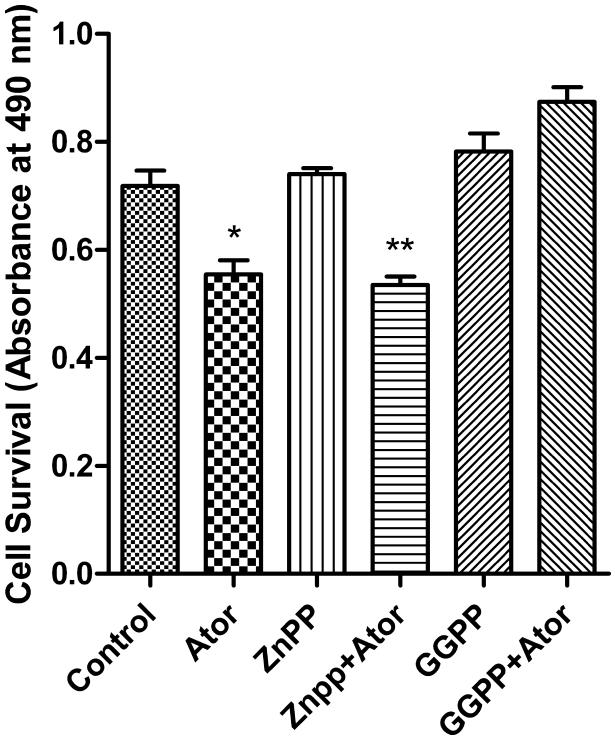

Fig. 4 Effect of ZnPP and GGPP on atorvastatin-induced cell death in PC-3 cells. PC-3 cells were treated with 10 μM atorvastatin (Ator) for 48 hrs in the absence or presence of 10 μM GGPP or ZnPP, and number of live cells was estimated by MTS cell proliferation assay as described in 'Materials and methods'. Results were expressed as absorbance at 490 nm (mean ± S.D.). Asterisks denote a difference from control that is significant at $*P < 0.05$ or $**P < 0.01$.

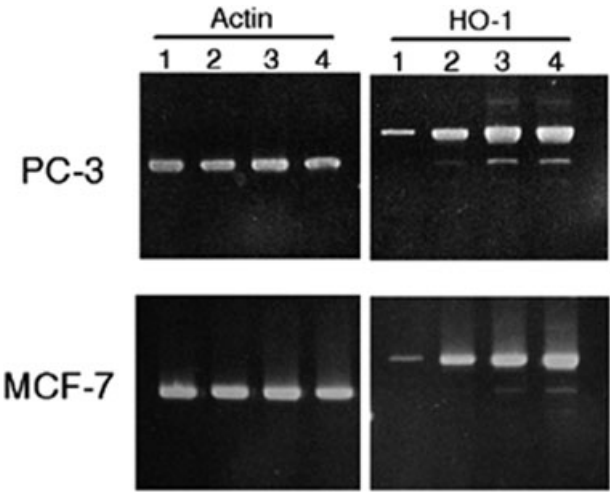

Fig. 5 Effect of ZnPP and GGPP on atorvastatin-induced up-regulation of HO-1 in PC-3 cells. PC-3 were treated with: (1) 10 μM GGPP, (2) 10 μM GGPP plus 10 μM atorvastatin, (3) 10 μM ZnPP or (4) 10 μM ZnPP plus 10 μM atorvastatin for 48 hrs. MCF-7 cells were similarly treated with 50 μM atorvastatin in the presence or absence of GGPP or ZnPP. Total RNA was isolated from these cells, and expression of β-actin and HO-1 was analysed by RT-PCR using gene-specific primers. The PCR products were analysed on 2% agarose gel.

Basal expression of HO-1 in PC-3 and MCF-7 cells was low, and overexpression of HO-1 in these cells was shown to inhibit cell proliferation and invasion [26, 27]. Therefore, it is likely that HO-1 is the mediator of atorvastatin-induced cell death in these cells. To investigate the role of HO-1 in atorvastatin-induced cell death, the effect of GGPP (an intermediate in the HMG-CoA reductase pathway) and ZnPP (an inhibitor of HO-1) on induction of cell death and regulation of HO-1 expression by atorvastatin was examined. When PC-3 cells were pre-incubated with GGPP, atorvastatin-induced cell death was abrogated (Fig. 4), similar to the results reported by other investigators using cerivastatin and other cancer cell lines [28, 29]. On the other hand, ZnPP did not protect the cells from atorvastatin-induced cell death (Fig. 4). For the regulation of HO-1 expression, no significant decrease of atorvastatin-induced HO-1 mRNA in the presence of GGPP was observed (Fig. 5, as compared to Fig. 2). Surprisingly, ZnPP was a stronger inducer of HO-1 than atorvastatin, and synergistic effect with atorvastatin could be seen in MCF-7 cells (Fig. 5).

Many ARE-like motifs are present in the promoter of human HO-1 gene. Six of these sites were found as clusters at E1 (−3928 bp) and E2 (−9069 bp) regions of the human HO-1 promoter, they are termed StRE1 through StRE6 [21]. StRE3 and StRE4 share identical core sequence, and StRE5 and StRE6 also share the same core sequence. The ARE site of human NQO1 promoter used in the initial experiments contains the same core sequence (5′-TGCTGAGTCA-3′) as in StRE3 and StRE4. To test the strength of theses ARE-like elements, StRE1, StRE2, StRE3 and StRE5 were subcloned into pGL3 vector and tested for induction by atorvastatin in PC-3 cells. As shown in Figure 6, StRE3 showed the highest induction level by atorvastatin, although these elements had different levels of relative luciferase activities due to different copy number of the response elements present in the luciferase-reporter constructs. Besides these StRE sites, other response elements, such as HSE [22], SREBP [23] and SP1 [24] sites have also been reported to be present in HO-1 promoter. Atorvastatin did not activate the HSE, SREBP and SP1 elements (Fig. 6).

Discussion

In this study, we demonstrated that atorvastatin up-regulated HO-1 expression in PC-3 and MCF-7 cells and that up-regulation was done through activation of the StREs of HO-1 promoter. We also demonstrated that pre-incubation of the cells with GGPP, an intermediate in the HMG-CoA reductase pathway, abrogated atorvastatin-induced apoptosis but not up-regulation of HO-1 expression, suggesting that induction of HO-1 gene by atorvastatin may be independent of GTPase activity. Furthermore, we demonstrated that ZnPP, an inhibitor of HO-1, also up-regulated

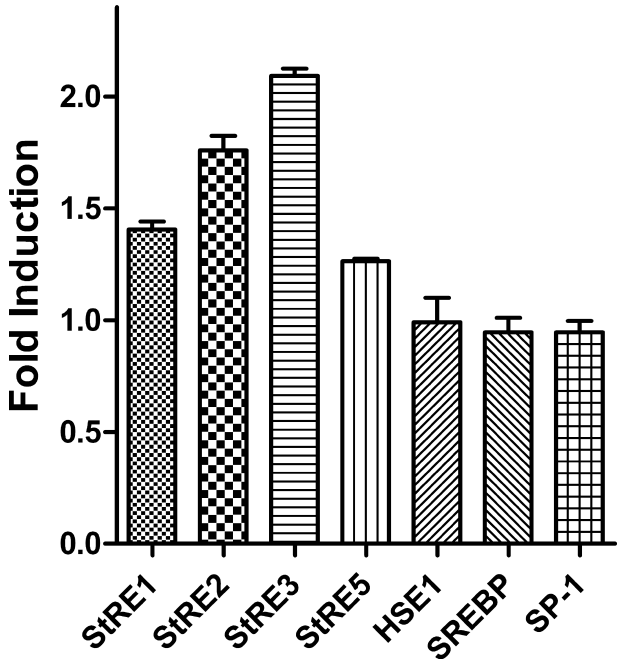

Fig. 6 Effect of atorvastatin on StRE1, StRE2, StRE3, StRE5, HSE, SREBP and SP1 elements of human HO-1 promoter in PC-3 cells. PC-3 cells were transfected with enhancer-luciferase reporter plasmid harbouring one of these elements, treated with 10 μM atorvastatin for 48 hrs and luciferase activities were determined as described in 'Materials and methods'. Results were expressed as 'fold induction' over vehicle (DMSO) control (mean ± S.D.).

HO-1 expression but did not protect cells from atorvastatin-induced apoptosis, suggesting that atorvastatin-induced apoptosis may be independent of HO-1 enzymatic activity. Up-regulation of HO-1 by statin has been reported in other cells including endothelial cells [30], macrophage [31], neuronal cells [32], liver [33], cultured human dental pulp cells [34] and vascular smooth muscle cells [35]. To the best of our knowledge, induction of HO-1 by statin in prostate and mammary cancer cells has not been reported by other investigators.

HO-1 expression can be induced by many inducers, and many regulatory pathways have been proposed [36]. Here we reported activation of StRE in the HO-1 promoter by atorvastatin. However, the transcription factor(s) involved are not known at the present moment. Nrf2 has been shown to play an important role in the up-regulation of HO-1 in the liver by simvastatin [33] and vascular smooth muscle cells by fluvastatin [37]. Interaction of Nrf2 and Bach1, two proteins of opposing action, with the StRE of human HO-1 promoter has also been reported [38]. However, the role of Bach1 in the activation of HO-1 by statin is presently unknown. Apart from the activation of StRE sites, statin has been shown to up-regulate HO-1 in macrophages *via* extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase G pathways [31]. It was recently reported that HO-1 was up-regulated through mRNA stabilization [39].

The role of HO-1 in cancer is controversial. On one hand, HO-1 catalyses the breakdown of heme, producing carbon monoxide, iron and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Carbon monoxide, biliverdin and bilirubin are antioxidants. Therefore, HO-1 is known to be a cytoprotective enzyme against oxidative stress [40]. HO-1 can be expressed at high levels in some tumour cells, and down-regulation of HO-1 by HO-1-shRNA or inhibition of the enzyme by specific inhibitor (OB-24) has been shown to inhibit proliferation of some hormone-refractory prostate cancer cells [41]. On the other hand, anti-proliferative and pro-apoptotic functions of HO-1 have been reported in prostate cancer [26], breast cancer [27] and oral cancer [42], although the mechanism of action is not known. HO-1 is an integral membrane protein of the smooth endoplasmic reticulum (sER). It is anchored on the sER *via* a transmembrane domain at the C-terminus with most part of the protein on the cytosolic side and a short C-terminus inside the lumen of sER. However, its nuclear localization has been demonstrated by immunocytochemical and Western blot analyses [43, 44]. This suggests that HO-1 may play a role in transcriptional activation of other genes. It was shown that removal of the C-terminus by proteolytic cleavage was responsible for the nuclear translocation of HO-1 [44]. HO-1 was shown to activate its own promoter and promote binding of nuclear proteins to the consensus sequences for activator protein 2 (AP-2), POU domain class 4 transcription factor, Brn3, and core-binding factor (CBF), suggesting that HO-1 plays an important role of modulating its own expression as well as the expression of other oxidant-responsive genes [44].

In summary, atorvastatin was found to induce apoptosis and up-regulation of HO-1 in PC-3 and MCF-7 cells. However, the role of HO-1 in atorvastatin-induced apoptosis in these cells remains to be studied.

Acknowledgement

This project was funded by a grant from Albert Einstein Society, AEHN, Philadelphia, PA (to S.C.M.K.).

Conflict of interest

The authors confirm that there are no conflicts of interest.

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