Silencing of Bcl-2 gene expression by siRNA transfection inhibits the protective effect of fluvastatin against cell apoptosis in human aortic endothelial cells

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Objective To study the protective effect of fluvastatin, one of the HMG-CoA reductase inhibitors (statins), against oxygen radical-induced oxidative damages in human aortic endothelial cell, and the role of Bcl-2 in this protection. Methods Human aortic endothelial cells with or without Bcl-2 siRNA transfection were subjected to 1-100 nM of fluvastatin and 100 μM hydrogen peroxide for 24 hours. Bcl-2 mRNA and protein expression were measured by Taqman quantitative PCR and Western blotting. Cell apoptosis was measured by normal and fluorescent microscopy and Cell Death Detection ELISA. Results In the Bcl-2-expressed cells, fluvastatin significantly reversed hydrogen peroxide-induced microscopic apoptosis and apoptotic DNA fragmentation, which were accompanied by a markedly upregulation of Bcl-2 expression by fluvastatin. However, the endothelial protection by fluvastatin was completely lost in Bcl-2 siRNA transfected cells. Conclusion Fluvastatin protects human endothelial cells against oxygen radical-induced cell apoptosis in vitro, and this protection seemed to be mediated in a Bcl-2 dependent pathway. (J Geriatr Cardiol 2008; 5:33-38)

Key Words fluvastatin, Bcl-2, gene silencing, apoptosis

Introduction

Fluvastatin is one of the 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors (statins) for the treatment of hypercholesterolemia. Its efficacy and safety have been established in numerous clinical trials. Emerging evidence now indicates that treatment with fluvastatin slows the progression of atherosclerotic chronic heart diseases and reduces the incidence of cardiovascular mortality in the secondary prevention setting. This effect of fluvastatin cannot be explained by simply lowering cholesterol and the nonlipid-related mechanisms (so-called “pleiotropic effects”) seem to contribute to a certain extent, and are probably linked to modulation of the mevalonate pathway. These properties include beneficial effects on vessel endothelial tissue; decrease of low-density lipoprotein oxidation and inflammation; ability to stabilize atherosclerotic plaques and perhaps promote regression; anti-thrombotic effects by inhibiting platelet aggregation and stimulation of fibrinolytic factors; and improvement of blood viscosity and flow. With these actions, statins may benefit the situation of long-term atherosclerotic plaque formation and the setting of acute coronary syndrome.

Endothelial dysfunction has been recognized as the cellular basis of cardiovascular disturbances. Integrity of endothelial layer of the vascular wall and activated endothelial functions such as abnormal secretion of cell adhesion molecules, nitric oxide and other inflammatory agents contribute to the onset of atherosclerotic plaque and disturbed vascular tone which in turn lead to increased risk of cardiovascular diseases. Oxidative stress is a well-known inducer of endothelial dysfunction, and also a risk factor of cardiovascular diseases. Therefore, it is important to seek some therapies that offer endothelial protection against oxidative damages in the prevention of cardiovascular diseases. Statins, especially fluvastatin, have been shown to have anti-oxidative effect in addition to their cholesterol-lowering property. Rikitake et al reported that fluvastatin treatment reduced vascular superoxide levels and plaque formation in cholesterol-fed rabbits. Suzumura et al demonstrated fluvastatin scavenging of hydroxyl radical and superoxide anion. In addition, fluvastatin has been shown to afford protection against DNA damage. However, the effect of fluvastatin on oxygen-radical-induced human endothelial oxidative damages and the mechanisms of this effect are still unclear.

Bcl-2, an integral membrane protein located in the cytoplasmic side of endoplasmic reticulum membrane, nuclear envelope and the outer membranes of the mitochondria, is a well-known pro-survival protein, which plays a beneficial role in preserving mitochondrial activities. The expression of Bcl-2 can be influenced by reactive oxygen species, cytokines or chemicals. Modulation of Bcl-2 expression, up-regulating or down-regulating, leads to improve or decrease
cellular protection against exogenous insults. Fluvastatin at relatively higher concentrations seemed to have adverse effect on cell growth and survival via a Bcl-2 dependent pathway. However, whether or not its protective effect against cell oxidative damages is Bcl-2 dependent is still obscure.

In this study, we used human aortic endothelial cells as an in vitro human endothelial cell model to investigate the potential protective effect of fluvastatin on oxygen radical (hydrogen peroxide)-induced cell apoptosis and proliferation interference, and investigate the influence of different Bcl-2 expression levels, normally expressed or silenced, on fluvastatin protection.

Materials and methods

Source of reagents

All reagents and chemicals were obtained from Sigma, UK unless otherwise stated. All reagents for tissue culture were obtained from Gibco Invitrogen, UK. The Bcl-2 siRNA/siAbTM Assay Kit was obtained from Upstate, USA. Fluvastatin was provided as a gift from Novartis, UK.

Cell culture

Primary HAoECs were purchased and cultured in Endothelial Cell Growth Media MV 2 (Promocell, Germany), which were supplemented with 5% fetal calf serum, 5.0 ng/ml epidermal growth factor, 0.5 ng/ml vascular endothelial growth factor, 10ng/ml basic fibroblast factor, 20 ng/ml R3 IGF-1, 50 ng/ml amphotericin B and 50 µg/ml gentamicin. This medium kit initially contained supplements of ascorbic acid and hydrocortisone, which were omitted for this study, and the concentration of D-glucose of this medium was 5%. Cells were kept in a humidified atmosphere of 5% CO2 in air at 37°C in the incubator and used at passage 2 to avoid age-dependent variations in apoptosis. Cells were then seeded at 37°C in the incubator and used at passage 2 to avoid age-dependent variations in apoptosis. Cells were then seeded at the density of 3.0×10⁴/ml and cultured without Bcl-2 siRNA transfection. All the cells were incubated at 37°C for 24 hours and then exposed for another 24 hours to fluvastatin at different doses immediately prior to 100 µM hydrogen peroxide (H₂O₂). Cell Bcl-2 expression, apoptosis and proliferation were subsequently determined using the methods stated below. All the experiments were performed in triplicates, and results confirmed by two repeated experiments.

Bcl-2 siRNA transfection

The Bcl-2 siRNA was transfected to the cells using the Lipofectamine method according to the manufacturer’s protocol (Invitrogen). Briefly, for each 10 cm² plate surface area, the mixture of 10 µl Bcl-2 siRNA and 10 µl Lipofectamine was incubated for 20 minutes at room temperature to allow the siRNA/Lipofectamine complexes to form before added to the cells, which were given 800 µl of serum/antibiotics-free medium after washed with the same medium twice. The final concentration of Bcl-2 siRNA was 100 nM.

Measurements of Bcl-2 expression

Taqman quantitative PCR for Bcl-2 mRNA expression

Cell total RNA was extracted using Trizol reagent and treated with DNase to remove genomic DNA. RNA was then reverse-transcribed to cDNA using M-MLV Reserve Transcriptase. The GeneAmp 5700 sequence detection system (Applied Biosystems, UK) for PCR was used to measure the relative level of gene expression quantitatively. Each sample was amplified with primers and probes for the target gene as well as the housekeeping gene, human β-glucuronidase (hGUS). The primers and probes were designed using Primer Express software (Applied Biosystems) and synthesized by MWG-Biotech (Germany). The sequences of primer set were submitted to a BLAST search at http://www.ncbi.nlm.nih.gov/BLAST/ to confirm their uniqueness. The internal probe was labelled at the 5’ end with the reporter fluorochrome 6-carboxyfluorescein (FAM) and at the 3’ end with the quencher fluorochrome 6-carboxytetramethylrhodamine (TAMRA). For Bcl-2, forward primer: 5’- TTG GCC CCC GGT GCT T -3’; reverse primer: 5’- CGG TTA TCG TAC CCC GTT CTC -3’; probe: 5’-AGC GTG CGC CAT CCT TCC CAG-3’. DEPC-treated water was used as a non-template control and non-reverse transcribed samples were used to confirm that positive results were not due to amplification of genomic DNA. The results were analyzed using GeneAmp 5700 software, and the baseline and threshold were set manually. The PCR cycle consisted of an initial cycle of 50°C for 2 minutes followed by 95°C for 10 minutes, then 50 repeated cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (primer annealing and extension). The relative expression of Bcl-2 mRNA in each treatment group was expressed as folds of the normal control.

Western blotting for Bcl-2 protein expression

Protein was obtained by homogenizing the cell pellet in 2× SDS sample buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol Blue, 200 mM α-mercaptoethanol) and separated on 10% SDS-PAGE gel using the Jencons vertical protein gel apparatus (SLS Laboratory Equipment) before transfer to a nitrocellulose membrane (0.45 mm pore size, Electra, BDH) using a semi-dry transfer system (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). The blot was then incubated with the primary antibody for human Bcl-2 (Anti-Bcl-2 clone 100, mouse monoclonal IgG, provided in the siRNA kit) and a secondary antibody conjugated with horseradish peroxidase (HRP) (1:1000 dilution, goat anti-mouse IgG-HRP, Bio-Rad). Visualization was car-
ried out using ECL enhanced chemiluminescence (Amersham, Amersham, UK) and an Epichemi II darkroom (UVP Lab Products, Cambridge, UK).

**Measurements of cell apoptosis**

**Normal and fluorescent microscopy** Morphological cell apoptosis was determined by normal and fluorescent microscopy. Hoechst 33342 (Sigma Aldrich, UK) at a final concentration of 1 µg/ml was added to the culture medium, which gave the cells blue nuclear fluorescence. Cell responses to different treatments were observed and recorded using the Leica DMIL fitted with a SPOT® digital camera (Diagnostic Instruments, USA) with a combination of Image Modulation Contrast for normal light and UV exposure with a wide band pass filter for fluorescence. Apoptotic and viable cells in different treatment groups were counted by trypan blue stain assay using a hemocytometer (Hausser Scientific, USA).

**Cell Death Detection ELISA assay** DNA fragmentation within apoptotic cells was determined by the Cell Death Detection ELISA assay (Roche Diagnostics Ltd, UK), employed according to the manufacturer’s protocol. Briefly, cells were lysed with 200 µl lysis buffer and centrifuged at 4,000 rpm for 10 minutes. Twenty microliter supernatant of each sample and 80 µl of the immunoreagent (1/20 anti-histone-biotin; 1/20 anti-DNA-POD; 18/20 incubation buffer) were added to each well of the streptavidin-coated microtiter plate (Anthos 2010, Anthos Labtech Instruments). After incubation and three times washes, 100 µl ABTs substrate solution was added. The absorbance was measured using a spectrophotometric microtiter plate (MTP) reader (Anthos 2010, Anthos Labtech Instruments) at the wavelength of 405 nm, and 490 nm was used as the reference wavelength.

**Cytotoxicity detection assay LDH** Necrosis was determined through the activity of lactate dehydrogenase (LDH) released from the cytosol; the Cytotoxicity Detection Kit (Roche Diagnostics Ltd, UK) was used according to the manufacturer’s protocol. Briefly, 100 µl culture supernatant of each sample was transferred into a 96-well plate, followed by adding 100 µl reaction mixture to each well. The samples were incubated for 20 minutes in the dark at room temperature and the absorbance was measured by the MTP reader at the wavelength of 492 nm with 602 nm as a reference.

**Statistical analysis**

Statistical analysis was performed on three replicates using ANOVA with Tukey’s post hoc analysis (SPSS version 11, SPSS UK Ltd, Surrey, UK). Data are expressed as mean ± standard deviation. Values of P<0.05 were considered statistically significant.

**Results**

**Bel-2 expression was upregulated by fluvastatin but silenced by siRNA transfection**

**Bel-2 mRNA expression** As shown in Figure 1, fluvastatin ranging 1-100 nM up-regulated Bel-2 mRNA expression by up to 18.51 folds compared with the normal control. The Bel-2 expression level was reduced by around 50% by H2O2, but in the presence of fluvastatin (10 nM and 100 nM), the Bel-2 mRNA level was elevated to 8.46 and 13.72 folds of the normal control respectively, not only restoring the decreased level by H2O2 but also inducing much higher level of Bel-2 expression than the normal control. Forty eight hours after Bel-2 siRNA transfection, the mRNA expression was silenced and not detectable with Taqman quantitative PCR in all the treatment groups (Figure 2A).

**Bel-2 protein expression** The Bel-2 protein was found to be expressed in the normal HAOECs and the expression band in fluvastatin treated group was stronger than the normal control, indicating an elevated expression level of Bel-2 in this group (Figure 2B). The Bel-2 protein bands were approximately 26 ± 10 kDa, corresponding to the band detected in the positive control of the Raji RIPA cell lysate, which was supplied by the Bel-2 siRNA/siAb™-
say Kit. In the Bcl-2 siRNA transfected cells, the Bcl-2 protein expressed was completely blocked and the protein bands were too weak to be seen (Figure 2B).

Effect of fluvastatin on H$_2$O$_2$-induced cell apoptosis in HAoECs with or without Bcl-2 siRNA transfection

Microscopic apoptosis In the normal HAoECs, many detached and floating apoptotic cells were present in 100 $\mu$M H$_2$O$_2$-treated group, while healthy cells which were in normal shape and stuck to the culture plates predominated in the normal control under light microscopy. In the fluvastatin co-treated group, fewer dead cells were observed than the H$_2$O$_2$ group. However, within the siRNA-transfected cells, fluvastatin co-treatment did not reduce the number of apoptotic cells caused by H$_2$O$_2$. As shown in Figure 3A, viable cell number counted by the trypan blue staining method decreased after 24 hours exposure to H$_2$O$_2$ alone, but it increased in combined treatment with fluvastatin (10 nM and 100 nM). On the contrary, apoptotic cell numbers increased with H$_2$O$_2$ and decreased following the treatments with fluvastatin and oxygen radicals. In the siRNA-transfected cells, fluvastatin did not reduce the apoptotic cell number caused by H$_2$O$_2$. Similar phenomena were obtained with fluorescent microscopy (Figure 3B). With Hoechst 33342 staining, all the cells gave out blue nuclear fluorescence and apoptotic cells were indicated by the condensed nuclear staining. Less apoptotic cells were observed within fluvastatin co-treated cells, but in the siRNA transfected HAoECs, the number of apoptotic cells was identical to the H$_2$O$_2$-treated group.

Apoptotic DNA fragmentation The cell apoptotic levels in each treatment group were quantified using the Cell Death Detection ELISA which measured the DNA fragmentation in apoptotic cells. In both normal and Bcl-2 siRNA transfected HUVECs, increased apoptotic DNA fragmentation was observed within H$_2$O$_2$-treated cells, indicating an elevation of apoptotic rate in these cells (Figure 3C). Fluvastatin at 10 nM and 100 nM significantly reduced DNA fragmentation levels in the cells without Bcl-2 gene silencing (Figure 3C, $P<0.05$). In the Bcl-2 siRNA transfected cells, fluvastatin administration did not cause reduction of apoptosis induced by H$_2$O$_2$ (Figure 3C). Necrosis was not detectable in the above treated groups (Figure 3D).

Discussion

In this study, we found fluvastatin prevented H$_2$O$_2$-induced apoptosis. This protective effect was mediated by the up-regulation of Bcl-2 expression. The inhibition of Bcl-2 expression by siRNA abolished the beneficial effect of fluvastatin. The involvement of Bcl-2 in H$_2$O$_2$-induced apoptosis gives a new insight on the mechanism of how the protective effects of fluvastatin may be mediated.

Primary cultured endothelial cells are particularly prone to oxidative damage and often used to study H$_2$O$_2$ induced apoptosis. Our result showed that H$_2$O$_2$ at 100 $\mu$M is critical for producing “pure” apoptotic cell death in HAoECs. This concentration of H$_2$O$_2$ is similar to the report (50–100 $\mu$m), but it may be not applicable for other endothelial cells due to different sensitivity, such as aortic endothelial cells that showed no significant cell death occurring at less than 200 $\mu$m. Although the oxidative stress-induced apoptosis has been described for several years, the underlying molecular mechanisms remain to be elucidated. The potential mechanisms of H$_2$O$_2$-induced endothelial cell apoptosis have been linked to activation of JNK activation, alteration of mitochondria function which is coupled to the redox signalling pathways, such as glutathione reductase-dependent glutathione redox-cycling, S-nitrosylation of thioredoxin.

Bcl-2 is a key protein in the regulation of cell apoptosis and proliferation, however, the involvement of Bcl-2 in the H$_2$O$_2$-induced vascular endothelial cell apoptosis is still not clear. Lee et al. reported that the expression of Bcl-2 protein in HUVECs was decreased in the presence of 500 mM H$_2$O$_2$, however we found this concentration (500 mM) is relatively high and a large amount of cells died due to necrosis, rather
than apoptosis, therefore it is difficult to give a good conclusion that the involvement of Bcl-2 in the high H$_2$O$_2$-induced apoptosis. In deed, our result shows the apoptotic concentration of H$_2$O$_2$ (100 mM) did decrease the mRNA level of Bcl-2, which probed with real-time PCR, indicating the Bcl-2 is involved in the H$_2$O$_2$-induced apoptosis. Using Bcl-2 siRNA to knockdown the endogenous Bcl-2 expression, the apoptotic cell death was increased.

Statins have been recommended as an effective class of lipid lowering drugs, however, statins at high concentration may induce cytotoxicity, such as skeletal cytotoxicity, or apoptosis. Like other statins, fluvastatin at micromole also induces apoptotic cell death in several cell types, such as HUVECs, rat pulmonary vein endothelial cells, cardiac myocytes, rat myoblasts, human CD4$^+$ T cells, human hepatocytes, vascular smooth muscle cells, lymphoblasts, breast cancer cells, and renal cancer. However, fluvastatin at low concentration can significantly protect the apoptotic cell death induced by H$_2$O$_2$. The mRNA and protein levels of Bcl-2 were significantly increased by fluvastatin, especially the Bcl-2 mRNA was more than ten times (~15 fold) increased in the groups treated with fluvastatin. This unexpected action may be a novel molecular mechanism of fluvastatin. Unfortunately we have not tested other statins to see if this is a class effect, but simvastatin at high concentration (30 mM) has been demonstrated to inhibit the expression of Bcl-2, and the effect was restored by mavalonate (13), suggesting statins may have different mechanisms or perhaps a biphasic regulation on Bcl-2 expression. When the Bcl-2 expression was inhibited by siRNA, fluvastatin lost its protective effect, suggesting that the Bcl-2 protein is an important new target of fluvastatin. The question how fluvastatin regulates the Bcl-2 expression is interesting and to be addressed in further study. To our knowledge, this is the first comprehensive study demonstrating the quantitative changes of Bcl-2 expression.
mRNA in the HUVEC apoptotic model induced by H2O2. Endogenous H2O2 can be generated by several enzymes in cells. Recent studies indicate that low-level ROS is necessary for the processes involved in both angiogenesis and endothelial cell survival. For example, VEGF-induced endothelial cell proliferation and migration were shown to be dependent on O2 generation. We also found that H2O2 at low concentration increased cell proliferation in HAOECs (data not shown), however, the major effect of H2O2 at 100 mM is apoptotic, and also the inhibited cell proliferation. Fluvastatin at low concentration protects the H2O2-induced cell proliferation inhibition, and this protective effect was abolished by Bcl-2 siRNA, suggesting that the effect of fluvastatin on cell proliferation under oxidative stress is mediated by Bcl-2 expression as well. Fluvastatin at high concentration can inhibit cell proliferation as shown in HAOECs of this study and of others in other cell types.14,23-27

In conclusion, hydrogen peroxide-induced cell apoptosis in HAOECs provides a good model for studying cell growth and death under oxidative stress. Fluvastatin is a potent protective agent against hydrogen peroxide-induced cell apoptosis, and Bcl-2 is a novel target for its protective effect.

References