Simvastatin-Induced Heme Oxygenase-1 Increases Apoptosis of Neuro 2A Cells in Response to Glucose Deprivation

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Heme oxygenase-1 (HO-1) has been suggested as an important mediator of the cholesterol-independent cytoprotection actions of statins, which may be of benefit for the treatment of degenerative neurological diseases and for reduction of infarct volume after cerebral ischemia. Overexpression of HO-1, however, has dual effects under oxidative stress, and the release of ferric iron from heme under these conditions may result in detrimental rather than cytoprotective effects. This study was designed to investigate the effect of simvastatin-induced HO-1 on Neuro 2A cells in response to glucose deprivation. We demonstrated that simvastatin induced a dose- and time-dependent upregulation of HO-1 protein expression in Neuro 2A cells. The induction of HO-1 after simvastatin treatment was mediated by nuclear factor erythroid 2–related factor 2 (Nrf2), which was expressed by Western blots of nuclear fractions and retarded complex formation in the electrophoretic mobility shift assay reaction. In addition, simvastatin activated the extracellular signal–regulated kinase and p38, but not the phosphorylation of c-Jun N-terminal kinase and Akt. Glucose deprivation in the cells pretreated with simvastatin induced more HO-1 expression, and the transcript could be decreased by small interfering RNA for Nrf2. This upregulation of HO-1 was significantly associated with increased apoptosis, manifested as expression at the protein level of 17-kDa cleaved caspase-3 and increased percentage of apoptotic cells shown by flow cytometry. The increased cleaved caspase-3 expression and percentage of apoptotic cells was significantly reduced by the HO inhibitor zinc protoporphyrin. Addition of the iron chelator desferrioxamine also resulted in blockade of the aggravated apoptosis, which implies that iron production from HO-1 activity may play an important role in the increased apoptosis in response to glucose deprivation in neuronal cells pretreated with simvastatin.

Key Words: apoptosis; desferrioxamine (DFO); glucose deprivation; heme oxygenase-1 (HO-1); nuclear factor erythroid 2–related factor 2–related factor 2 (Nrf2); simvastatin; zinc protoporphyrin (ZnPP).
Nrf2 protein levels barely detectable under normal conditions. Oxidant insults promote the dissociation of the Nrf2–Keap1 complex, resulting in stabilization of the Nrf2 protein, translocation to the nucleus, and upregulation of the phase II gene (Cullinan et al., 2004; Itoh et al., 1999; Motohashi and Yamamoto, 2004).

Many reports have demonstrated the potent antioxidant, anti-inflammatoty and antiapoptotic activity of the heme-derived metabolites generated by HO-1 catalysis on vascular endothelium and nerve cells (Chen et al., 2000; Moon et al., 2003; Otterbein and Choi, 2000). However, some reports have suggested a duality of effects of HO-1 overexpression in oxidative stress (da Silva et al., 1996; Dennery et al., 1997; Suttner and Dennery, 1999; Suttner et al., 1999). The release of ferric iron from the porphyrin ring of heme during this reaction may result in detrimental effects, because this form of iron is known to catalyze oxidative reactions (Gutteridge et al., 1982). In addition, reversal of HO-1 related cytoprotection with increased expression is due to accumulation of the reactive iron, which may have potentially damaging effects from the generation of free radicals with significant oxygen cytotoxicity (Suttner and Dennery, 1999).

HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors, also known as statins, are lipid-lowering agents that decrease cardiovascular morbidity and mortality by lowering cholesterol levels and subsequently atherosclerotic changes of blood vessels (Maron et al., 2000). With the ability to cross the blood–brain barrier (Guillot et al., 1993), statins are also promising candidates for their potentially neuroprotective effects in neurological diseases such as Alzheimer’s disease and other forms of dementia (Jick et al., 2000; Sparks et al., 2005; Vaughan, 2003; Wolozin et al., 2000) as protective effects against cerebral ischemia (Amin-Hanjani et al., 2001; Endres et al., 1998; Laufs et al., 2002). Simvastatin has been reported to be the most effective statin in reducing infarct size and in improving the clinical outcome after focal cerebral ischemia in mice (Endres et al., 1998). After statin treatment, HO-1 activity increases in the brain, liver, lung, and heart tissue of the mouse 24 h later (Hsu et al., 2006), and HO-1 induction in the brain has been suggested as the pathway of cholesterol-independent cytoprotective effects of statins in reducing stroke-related ischemic injury (Maines, 2002). HO-1 induction has also been proposed to explain the pleiotropic antioxidant, anti-inflammatory actions of statins on the vessels (Grosser et al., 2004a,b; Lee et al., 2004). Although the neuroprotective effects of different statins have been demonstrated in some experimental settings, it has also been reported that treatment of astrocytes with atorvastatin and simvastatin induces a time- and dose-dependent apoptosis (Marz et al., 2007), and lovastatin can suppress cell growth by inducing apoptosis of neuroblasts in a dose- and time-dependent manner by inhibition of isoprenoid biosynthesis (Garcia-Roman et al., 2001). Therefore, definitive conclusions regarding the effect of statins, particularly the role of statin-induced HO-1, on neuronal cells still cannot be drawn.

We designed this study to investigate the role of Nrf2 in HO-1 induction with simvastatin treatment of cultured Neuro 2A cells, and also to explore the effect of the induced HO-1 against the oxidative stress of glucose deprivation. Our results demonstrated that there is an involvement of Nrf2 in mediating the upregulation of HO-1 in response to simvastatin treatment. However, the induced HO-1 failed to have a cytoprotective effect on the neuronal cells and rather resulted in an exaggerated increase in cleaved caspase-3 expression and apoptosis after glucose deprivation. Furthermore, blockade of HO-1 with zinc protoporphyrin (ZnP), an HO competitive inhibitor, and addition of the iron chelator desferrioxamine (DFO) reduced the exaggerated apoptosis, implying that downstream iron formation from HO-1 activity might play a role in the exaggerated apoptosis response.

**MATERIALS AND METHODS**

**Materials.** Minimum Essential Medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY). Goat anti-rabbit HO-1 polyclonal antibody and goat anti-mouse β-actin monoclonal antibody were purchased from Stressgen (Stressgen, Victoria, Canada) and Chemicon (Chemicon, Temecula, CA). Anti-Nrf2, anti-mouse, and anti-rabbit IgG-conjugated horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nrf2 small interfering RNA (siRNA) and its transfection kit were obtained from Ambion (Austin, TX). Anti-caspase-3 (35 and 17 kDa), anti–phospho-ERK (extracellular signal–regulated kinase) (p44/p42), anti–phospho-JNK (c-Jun N-terminal kinase) (Thr183/Tyr185), anti–phospho-p38 (Thr180/Tyr182), anti–phospho-Akt (ser473), and the related control antibodies were purchased from Cell Signaling Technology (Beverly, MA), fluorescein isothiocyanate (FITC)–conjugated Annexin V and propidium iodide (PI) for flow cytometry were purchased from MBL (Nagoya, Japan). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

**Cell culture.** A mouse neuroblastoma cell line, Neuro 2A, has been widely used to investigate the neurotoxic effects of various compounds and their associated mechanisms (Johnson et al., 2005; LePage et al., 2005; Lin et al., 2006). Neuro 2A cells were obtained from Bioresource Collection and Research Center, Taiwan. Neuro 2A cells were cultured in MEM supplemented with 10% FBS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. Cells were kept in an atmosphere composed of 5% CO2/95% O2 at 37°C. During glucose deprivation, cells were cultured in Earle’s Balanced Salt Solution.

**Cell viability assay.** Approximately 5000 Neuro 2A cells were grown in 100 μl of 10% FBS-supplemented MEM medium in 96-well flat-bottomed plates overnight. Treated cells were exposed to simvastatin (50μM) that was dissolved in dimethyl sulfoxide (DMSO) before being added to the medium. Cell viability was analyzed by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma) assay in three replicates. After 2 days in culture, attached cells were incubated with MTT (0.5 mg/ml, 3 h) and subsequently solubilized in DMSO. The absorbancy at 490 nm was then measured using a microplate reader.

**Immunocytochemistry.** The Neuro 2A cells were fixed with 4% formaldehyde and permeabilized with EtOH/CH3COOH (95:1). After being blocked with 5% bovine serum albumin, the fixed cells were incubated with anti–HO-1 antibody. The specimens were then incubated with secondary...
antibody conjugated with fluorescein and viewed under a Nikon TE300 fluorescence microscope.

Analysis of HO-1 messenger RNA levels. Using semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis, HO-1 messenger RNA (mRNA) analysis was performed in cells treated with simvastatin and/or glucose deprivation in the presence or absence of Nrf2 siRNA. The primer sets for detecting mouse HO-1 were sense primer, 5'-ATGGAGGCCGCA- CAGGTC-3', and antisense primer, 5'-TGCGGCCCATACCAGAA-3'. Three rodent 21-mer Nrf2 siRNA oligonucleotide were used as follows: 5'-GGACUAUGACUGGUAAAATT-3' (no. 68333), 5'-GCCGCUAA- GAGGCUCACUn-3' (no. 156499), 5'-GCCGCUUAGAGGCUCACUn-3' (no. 156500), with targeted sequences corresponded to bp 365–383, 1012–1030, and 1552–1570 of the Nrf2-coding region, respectively. Subsequently, the most effective target no. 156499 was used in the experiment for knocking-down Nrf2 expression. Transfection with the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA nucleotide obtained from Ambion (catalog number AM4624) with successful knock-down of GAPDH transcript was used to verify high efficiency of delivery of the oligonucleotide into the cells. Neuro 2A cells were plated in 24-well plates at 6×10^4 cells and transfected with a Nrf2 siRNA in concentrations of 10, 30, and 50nM or with a scramble control nonsilencing silencer siRNA (catalog number AM4611, Ambion) using a lipofectamine reagent mixed with 560 l of Opti-MEM I (Invitrogen) according to the manufacturer’s instructions. Total RNA, which was extracted from treated cells by applying 500 l of RNAzol directly onto the cells with the addition of DNase digestion steps, was measured by measuring absorption at 260/280 nm, and subjected to RT-PCR analysis. Total RNA was reverse transcribed using oligo-dT primers and RNase H reverse-transcriptase (Superscript II; Invitrogen) with 1 μg total RNA per sample. Quantification of HO-1 mRNA content was performed using a charge-coupled device camera (FluoChem 8900 imaging system; Alpha Innotech, San Leandro, US). The intensity of each signal was transferred into digital data with auto-background subtraction during spot density analysis using AlphaEaseFC software.

Immunoblotting. Fifty micrograms of cell lysate was resolved on 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, the proteins were electrotransfered to polyvinylidene difluoride membranes, blocked with 5% skim milk in Tween-20/phosphate-buffered saline, and incubated with the appropriate antibodies: anti–HO-1 (1:2000), anti–phospho-ERK, anti-ERK, anti–phospho-INK, anti-INK, anti–phospho-p38, anti-p38, anti–phospho-Akt, anti-Akt, anti-Nrf2, and anti–caspase-3 (1:1000). Anti-Nrf2 antibody was used in the blotting with nuclear extracts. The blots were then incubated with HRP-conjugated secondary antibodies. The protein bands were measured with FluorChem 8900 imaging system and the AlphaEaseFC software described above.

Electrophoretic mobility shift assays. Gel shift analysis was performed with 5 μg of nuclear proteins incubated for 20 min at room temperature with a 20 fmol of Nrf2 oligonucleotide probe (5'-TTTTATGCTGTCATGTTTT-3'). The DNA protein complexes were resolved on 4% polyacrylamide gel electrophoresis and visualized with FluoroChem 8900 imaging system. For supershift assays, the nuclear extracts were incubated with antibody Nrf2 at room temperature for 20 min at 25°C before the addition of the biotin-labeled DNA oligonucleotide.

Flow cytometry. A total of 2×10^5 cells per well of the Neuro 2A were seeded into six-well microplates for 24 h incubation at 37°C. Different groups of cells treated with or without simvastatin in the presence or absence of glucose deprivation and addition of HO inhibitor ZnPP were used in the experiment. Cells were trypsinized and gently washed once with medium followed by washing with PBS before resuspension in 85 μl of binding buffer. Double staining was performed with 10 μl of Annexin V–FITC and 5 μl of PI added to the resuspended cells. After incubation at room temperature for 15 min in the dark, 400 μl of binding buffer was added to the cell suspension. Measurement of the cell sample was performed by flow cytometry (EPICS ALTRA, Beckman Coulter, Miami, US).

RESULTS

Simvastatin-Induced Expression of HO-1

After treatment with increasing concentration of simvastatin for 24 h, expression of HO-1 protein was noted in the cytosol of Neuro 2A cells by immunocytochemistry (Fig. 1), with concentration-dependent increases in HO-1 protein occurring in the blots with between 50 and 100μM of simvastatin (Fig. 2A). There was a fourfold and fivefold increase of HO-1 in the presence of 50 and 80μM of simvastatin, respectively. Moreover, the treatment with 50μM simvastatin resulted in time-dependent upregulation of HO-1 transcript and protein. HO-1 mRNA levels were apparent as early as 3 h after treatment and this was followed by an increase in HO-1 protein levels at 6 h. HO-1 mRNA peaked at 6 h (> 100-fold) and significantly reduced by 24 h. HO-1 protein expression was induced and lasted for at least 24 h (Fig. 2B).

Involvement of Nrf2 in Simvastatin-Induced HO-1 Expression

Glucose deprivation alone for 24 h induced expression of Nrf2 protein in the nucleus within 2 h and lasted for at least 6 h.
and remarkably reduced by 24 h (Fig. 3A). As a positive control, cells were also treated with the 26S proteasome inhibitor MG132 alone. This blocked proteolysis and resulted in a large accumulation of Nrf2 protein within 3 h. However, activation of Nrf2 after glucose deprivation did not induce significant HO-1 protein expression (Fig. 3A). Simvastatin in 50μM concentration induced expression of Nrf2 protein, measured by Western blots of nuclear fractions (Fig. 3B). Accumulation of Nrf2 in the nucleus was detected within 0.5 h after simvastatin treatment, preceding increased HO-1 mRNA, which typically occurred after 3 h. The expression of Nrf2 lasted for at least 6 h, and disappeared by 24 h. The Nrf2 transcription factor has a relatively short half-life and has been implicated in the regulation of HO-1 gene expression. Of note, combined treatment with the proteasome inhibitor MG132 and simvastatin did not significantly increase Nrf2 levels over those obtained with each drug separately, suggesting that simvastatin prevents degradation of Nrf2 via the ubiquitin-proteasome pathway. Therefore, it is likely that a reduction in Nrf2 degradation activity results in Nrf2 accumulation after simvastatin treatment.

EMSA reactions were performed with nuclear extracts from cells exposed to simvastatin or MG132. As shown in Figure 4, formed retarded complexes were significantly increased in intensity upon treatment with simvastatin or MG132. When the EMSA reaction was conducted in the presence of a competitive cold probe, the intensity of this complex dropped significantly, indicating the presence of Nrf2 in the complex. When Nrf2

FIG. 2. (A) Concentration-dependent increases in HO-1 protein occurred after treatment with between 50 and 100μM simvastatin for 24 h. (B) Treatment with 50μM simvastatin resulted in a time-dependent upregulation of HO-1 transcript and protein expression. The induction of HO-1 by simvastatin was evident as early as 6 h, and lasted for at least 24 h; 50μM hemin for 1 h was used to induce HO-1 as a positive control. Each bar represents the mean ± SD of three independently performed experiments; *p < 0.05 versus control.

FIG. 3. (A) Glucose deprivation alone for 24 h induced nuclear Nrf2 expression, when compared with anti–protein-disulfide isomerase (PDI) antibody used for normalization, but there was no induction of HO-1 protein expression. Cells were also treated with 20μM proteasome inhibitor MG132 as a positive control (GD: glucose deprivation; +: positive control for HO-1 protein); (B) simvastatin at 50μM concentration induced increases in nuclear Nrf2 protein, that was detected within 0.5 h and lasted for at least 6 h after treatment. Combined treatment with MG132 and simvastatin did not significantly increase Nrf2 levels over those obtained with each drug separately. Each bar represents the mean ± SD of three independently performed experiments.
Antibody was added, an uppershifted band was detected in the reaction (Fig. 4, arrow).

Significantly more HO-1 expression after glucose deprivation was noted in the cells pretreated with simvastatin 50 μM for 24 h than in those treated with simvastatin alone (Fig. 5A, lane 4). This increased HO-1 expression was effectively inhibited by 10μM ZnPP (Fig. 5A). We also conducted experiments of Nrf2 knock-down by using siRNA to investigate whether HO-1 expression would be inhibited (Fig. 5B). Transfection with the positive control GAPDH siRNA nucleotides obtained from Ambion (catalog number AM4624) with successful knock-down of GAPDH transcript was used firstly to verify the high efficiency of delivery of the oligonucleotides into the cells (Fig. 5B, upper panel). Significantly more HO-1 mRNA expression after glucose deprivation was noted in the cells pretreated with simvastatin 50μM for 24 h. In addition, the stimulation of HO-1 expression is dependent on Nrf2 activation because it was completely prevented by siRNA against Nrf2 in concentrations from 10 to 50nM (Fig. 5B, lower panel). In contrast, the scramble siRNA in 30nM concentration, which was used as a negative control, had no effect on the expression of neither GAPDH nor HO-1 mRNA, respectively (Fig. 5B).

**Simvastatin Activates p38 and ERK**

We analyzed the effect of simvastatin on the three MAPK cascades leading to activation of ERK, JNK, p38 and on the survival pathway represented by the Akt kinases. As shown in Figure 3, simvastatin activated the ERK and p38 pathways with different kinetics. Activation of ERK was observed immediately after 10 min of stimulation and lasted for at least 2 h, whereas activation of p38 was observed after 60 min and increased at 2 h. Conversely, no phosphorylation of JNK and Akt was detected after simvastatin treatment (Fig. 6).
Simvastatin Increases Cleaved Caspase-3 Expression during Glucose Deprivation

Western blots were used to examine the cleavage of caspase-3 in response to simvastatin and/or glucose deprivation. This showed that accumulation of the cleaved caspase-3 protein, an active 17-kDa proteolytic form that induces apoptosis, was strongly increased 24 h later in the cultured Neuro 2A cells treated with simvastatin plus glucose deprivation but not in the cells treated with simvastatin or glucose deprivation alone. The increased cleaved caspase-3 expression after simvastatin and glucose deprivation treatment could be effectively reduced by 10μM of ZnPP (Fig. 7).

Simvastatin Increases Apoptosis after Glucose Deprivation

Quantification of apoptotic cells was carried out by double labeling for Annexin V-Fluos and PI in the cultured Neuro 2A cells and flow cytometry (Fig. 8A). When compared with controls, the percentage of Annexin V+/PI- apoptotic cells was significantly increased in cells treated with 50μM simvastatin only for 24 h or with 100μM DFO for 5 h, but not in the cells treated with glucose deprivation alone (Figs. 8A and 8B). In addition, a ninefold increase in apoptotic cells was detected after glucose deprivation in the Neuro 2A cells pretreated with simvastatin 50μM for 24 h compared with those without simvastatin pretreatment. Nearly half of the cultured cells underwent programmed cell death. Furthermore, the increased numbers of apoptotic cells induced by treatment with simvastatin plus glucose deprivation were significantly reduced by 10μM ZnPP as well as DFO in concentrations of 10, 50, and 100μM. However, addition of ZnPP or an iron chelator in different concentrations failed to return the level of apoptosis in the cultured cells to normal (Figs. 8A and 8B). These results demonstrate that HO-1 expression and active iron accumulation play important roles in inducing apoptosis of Neuro 2A cells in response to simvastatin and glucose deprivation treatment.

Effect of Simvastatin on Neuro 2A Cell Growth

To evaluate the effects of simvastatin and glucose deprivation on Neuro 2A cell growth (the total number of cells/dish), cell viability was analyzed by the MTT assay (Fig. 9) and showed the percentage of viable cells sextupled after exposure of Neuro 2A cells (time 0) to medium containing 10% FBS alone for 48 h, but significantly decreased by the simvastatin treatment in the presence or absence of glucose deprivation. Time course experiments showed that in comparison with the control cells, which grew gradually in the medium, simvastatin in 50μM concentration decreased Neuro 2A cell growth, and glucose deprivation 24 h did not cause significant decrease in cell growth. After 72 h, the percentage of viable cells was significantly decreased in those cells received simvastatin pretreatment plus glucose deprivation than...
those other three groups, including those received simvastatin treatment alone. Although nearly half of the cultured cells underwent programmed cell death was noted in those cells received simvastatin plus glucose deprivation from study by the flow cytometry, the difference between those received simvastatin treatment plus glucose deprivation and those received simvastatin alone was not significant at 48 h, probably because at this time point the MTT assay was not able to differentiate the early apoptotic cells from the viable cells.

**DISCUSSION**

In this study, our results demonstrate that simvastatin induces HO-1 expression in Neuro 2A cells in a dose- and time-dependent manner. After simvastatin treatment, Nrf2 was translocated into the nucleus and expressed earlier than the expression of HO-1 transcript and subsequent protein expression. Combined treatment with the proteasome inhibitor MG132 and simvastatin did not significantly increase Nrf2 levels over those obtained with simvastatin or MG132 alone, suggesting that simvastatin prevents degradation of Nrf2 via the ubiquitin-proteasome pathway. In contrast, although glucose deprivation for 24 h induced Nrf2 activation but was not responsible for inducing significant HO-1 protein expression, there may be different downstream signal pathways controlled by the activated Nrf2 after glucose deprivation and exposure to simvastatin. However, there was significantly more HO-1 expression after glucose deprivation in the cells treated with simvastatin. The increased upregulation of HO-1 by simvastatin plus glucose deprivation was effectively inhibited by the HO inhibitor ZnPP and siRNA for Nrf2. These above experiments suggest that simvastatin induces HO-1 expression by an Nrf2 protein mediated pathway. The mechanism by
which Nrf2 is liberated from the Nrf2–Keap1 complex may involve stabilization of Nrf2 protein, which has a short half-life and is degraded via the ubiquitin–proteasome pathway, and phosphorylation of Nrf2 (Nguyen et al., 2003). Where there was a role of phosphorylation of Nrf2 in the Nrf2 activation and the nuclear import and export signals in control of Nrf2 in the neuronal cells treated with simvastatin and/or glucose deprivation require to be elucidated in further investigation. There are three major MAPK pathways represented by the kinase cascade leading to activation of ERK, JNK, and p38, which have been suggested to be involved to some extent in the upregulation of HO-1 expression in response to diverse stimuli. For example, carnosol appears to regulate HO-1 expression via a mechanism involving Nrf2 and activation of ERK, JNK, and p38 pathways (Martin et al., 2004), sodium arsenite activates HO-1 in primary cultures of rat hepatocytes through JNK and p38 pathways, but not the ERK pathway (Kietzmann et al., 2003), curcumin upregulates HO-1 via the Nrf2 and p38 pathway in porcine renal epithelial cells (Balogun et al., 2003), cadmium induces HO-1 via sequential activation of the p38 and Nrf2, but not the MAPK pathways involving ERK and JNK (Alam et al., 2000), and arsenite induces HO-1 expression via the ERK and p38 pathways in chick embryo liver cells (Lu et al., 2000). Our results suggest simvastatin treatment can induce phosphorylation of ERK and p38, but not JNK, in Neuro 2A cells. However, the relationship between the phosphorylation of ERK and p38 and the upregulation of HO-1 expression required further evidence to clarify. In addition, the expression level of p-Akt that may mediate the antiapoptotic effects of statins (Contreras et al., 2002; Dimmeler et al., 2001; Llevadot et al., 2001; Sattler et al., 2005) was unchanged, indicating that phosphorylation of Akt does not play a role.

HO-1 has been generally reported to protect against cell death (Chen et al., 2000; Moon et al., 2003; Otterbein and Choi, 2000). Cells from mice with a target deletion of HO-1 were much more sensitive to apoptosis induced by serum deprivation, an effect that was greatly reduced by overexpression of HO-1 (Ferris et al., 1999). Nevertheless, it has also been reported that induction of HO-1 by different dietary polyphenol curcumin or epigallocatechin-2-gallate does not protect breast tumor cells or lymphoblasts, respectively, from apoptosis (Andreadi et al., 2006). In fact, exaggerated HO-1 activity may be detrimental to cells because in addition to the formation of bile pigments and CO, iron is released from the degradation of heme via HO. Iron is well known to mediate reactions leading to the formation of hydroxyl radicals and cellular programmed death. When neurons were treated with 200μM of FeSO₄ for 1 h, there were 50.6% of cells underwent apoptosis 36 h later (Zhang et al., 2003). Iron accumulation is thought to be responsible for the reversal of the cytoprotective effect during overexpression of HO-1 (Suttnner and Denney, 1999). Simvastatin has also been reported to inhibit proliferation of vascular smooth muscle cells as a possible consequence of increased HO-1 activity (Lee et al., 2004).

Overexpression of HO-1 after transfection with adenovirus containing the rat HO-1 gene stimulates vascular smooth muscle cell apoptosis in association with a marked increase in the cellular level of the proapoptotic protein p53 (Liu et al., 2002). In the present study, simvastatin-induced HO-1 failed to play a cytoprotective role like the pleiotropic effect it provides to endothelial cells (Grosser et al., 2004a,b) or to the vascular system (Lee et al., 2004). In contrast, it actually aggravated the apoptosis of neuronal cells after glucose deprivation, with about a ninefold increase in apoptosis detected in those neuronal cells pretreated with simvastatin. Inhibition of induced HO-1 by ZnPP as well as chelation of the cellular iron with DFO reduced the increased programmed cell death, implying that HO-1 expression and iron formation were involved in the exaggerated apoptosis. However, the addition of the HO inhibitor or iron chelator in different concentrations failed to bring down the level of apoptosis in the cultured cells to a normal level, indicating that there may be some other mechanisms contributing to increased apoptosis of neuronal cells during glucose deprivation after simvastatin treatment. This result was different from that reported by Liu et al. (2002), which suggested that iron was not involved in the stimulated apoptosis in vascular smooth muscle cells, because the iron chelator DFO failed to abrogate the apoptotic effect. Lovastatin has been reported to decrease cell growth by inducing apoptosis of proliferating neuronal cells by blocking the effect of HMG-CoA reductase inhibitor on downstream mevalonate production, which is required for proliferating neuronal cells for cell growth (Garcia-Roman et al., 2001).

Therapies involving the induction of overexpression of HO-1 must be considered carefully before implementation. In most reports, HO-1 appears involved in protective mechanisms against vascular injury. In two other studies, Grosser et al. demonstrated that the induction of HO-1 expression by lovastatin and simvastatin (Grosser et al., 2004b) as well as by rosvastatin (Grosser et al., 2004a) at 100–300μM concentrations had cytoprotective actions in ECV304 and EA by 926 endothelial cells. In the present study, high concentrations of simvastatin beyond the pharmacological dosage, that is, 50–100μM, were needed to induce HO-1 expression in neuronal cells. Although HO-1 induction in the brain reduces stroke-related ischemic injury and might therefore contribute to the main neuroprotective actions of statins (Kretz et al., 2006), some reports have suggested that statins could act against stroke-related ischemic injury or other pathological alterations to the brain such as subarachnoid hemorrhage, ischemia, and traumatic brain injury and in human neurodegenerative diseases (Amin-Hanjani et al., 2001; Endres et al., 1998; Jick et al., 2000; Vaughan, 2003; Wolozin et al., 2000). However, it is important to assess whether these protective effects of statins are mediated through the vascular system or neurons themselves. As the response of neuronal cells to statins might be quite different to that of endothelial cells, concerns have to be raised about interpreting the physiological relevance of experiments.
in vitro or in vivo on different cell types and with different concentrations of statins. In addition, the response of primary cultured neuronal cells, not only cultured cell lines, to statin treatment is worth further investigation.

In summary, simvastatin stimulates HO-1 expression in Neuro 2A cells by Nrf2 protein. Simvastatin-induced HO-1 was not associated with protection of neuronal cells against glucose deprivation, but instead resulted in exaggerated apoptosis of the cells. Inhibition of the HO-1 upregulation or chelation of iron was able to reduce, but not completely inhibit, the apoptosis, implying that downstream iron formation from HO-1 activity plays a role in the response.

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REFERENCES


