Statin-Induced Heme Oxygenase-1 Increases NF-κB Activation and Oxygen Radical Production in Cultured Neuronal Cells Exposed to Lipopolysaccharide

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With potentially neuroprotective properties, heme oxygenase-1 (HO-1) has been suggested to be the main mediator of cholesterol-independent anti-inflammatory and antioxidant actions of statins. However, we had demonstrated that simvastatin-induced HO-1 increased apoptosis of Neuro 2A cells in glucose deprivation, and iron production from HO-1 activity may be responsible for the toxicity. This study was designed to explore the effect of simvastatin-induced HO-1 on cultured Neuro 2A and C6 cells exposed to lipopolysaccharide (LPS). We found that the HO-1 upregulation was significantly associated with increased nuclear factor kappa B (NF-κB) activation, manifested as IκBα phosphorylation and p65 nuclear translocation, as well as increased production of superoxides. Inhibition of the induced HO-1 by zinc protoporphyrin reduced the increased NF-κB activation and superoxides production. RNA interference with HO-1 siRNA reduced the expression of HO-1 transcripts and protein as well as oxygen radical production. Addition of the iron chelator desferrioxamine to reduce the accumulation of ferric iron from heme by HO-1 resulted in blockade of the aggravated oxygen radical production. There was no significant effect on production of oxygen radicals under these conditions in the presence of a CO donor (RuCO) or a CO scavenger (hemoglobin). In addition, the viable cells were significantly decreased in 48 h in those cells receiving simvastatin pretreatment plus LPS compared to those in control or exposed to simvastatin or LPS alone. This study revealed that simvastatin-induced HO-1 led to increased NF-κB activation and superoxides production in the neuronal cells when exposed to LPS, and iron production may play a role in such a response.

Key Words: heme oxygenase-1 (HO-1); lipopolysaccharide (LPS); nuclear factor kappa B (NF-κB); RNA interference (RNAi); simvastatin; zinc protoporphyrin (ZnPP).

HMG-CoA reductase inhibitors, also known as statins, are lipid-lowering agents that are widely used in medical practice (Maron et al., 2000). Today, it is generally accepted that mechanisms beyond the reduction of plasma cholesterol contribute significantly to the anti-atherogenic and tissue protective properties of statins (Massy et al., 1996; Wierzbicki et al., 2003). These pleiotropic, cholesterol-independent effects observed in the presence of statins include reduction of pro-inflammatory signaling such as cytokine and oxygen radical formation (Grosser et al., 2004b; Hayashi et al., 2005; Laufs et al., 2002; Lee et al., 2004). Some studies have demonstrated that statins lead to antioxidant defense protein heme oxygenase-1 (HO-1) promoter activation, transcription and protein accumulation and that this may explain the pleiotropic antioxidant, anti-inflammatory actions of statins (Grosser et al., 2004a,b; Lee et al., 2004). HO-1 is an inducible enzyme that catalyzes the degradation of heme to biliverdin, with the concurrent release of iron and carbon monoxide (CO). Over the past few years, numerous studies have revealed the important function of HO-1 as a cytoprotective defense mechanism against oxidative insults through the antioxidant activities of biliverdin and its metabolite, bilirubin, and the anti-inflammatory action of CO (Chen et al., 2000; Otterbein and Choi, 2000). HO-1 antisense and knockout studies and clinical investigations of HO-1 promoter polymorphisms have clearly shown that HO-1 assumes a central role in cellular antioxidant defense and, specifically, in vascular protection (Chen et al., 2002; Otterbein et al., 2003; Yet et al., 2003). Elevated HO-1 protein levels have been associated with reduction of free radical formation in endothelial cells after simvastatin and lovastatin treatment (Grosser et al., 2004b). Simvastatin-induced inhibition of inflammatory responses mediated through HO-1 can lead to prevention of lipopolysaccharide (LPS)–induced IκB degradation in human aorta vascular smooth muscle cells and the ensuing nuclear translocation of nuclear factor kappa B (NF-κB) p65 (Lee et al., 2004). Treatment with additional zinc protoporphyrin (ZnPP), an HO competitive
inhibitor, abolishes the preventive effect of simvastatin on NF-κB activation (Lee et al., 2004).

HO-1 activity in the brain, liver, lung, and heart tissue increases 24 h after statin treatment (Hsu et al., 2006). Cytoprotective and anti-inflammatory actions of HO-1 outside the vasculature have also been documented in various tissues, including the heart, kidney, and neuronal cells (Immenschuh and Ramadori, 2000; Otterbein and Choi, 2000; Polte et al., 2002). However, before introducing the routine use of statins to induce HO-1 and act as cytoprotective agents for various organs, note that some reports have suggested a possible duality of effects of HO-1 overexpression in oxidative stress (da Silva et al., 1996; Denner et al., 1997; Suttner and Denner, 1999; Suttner et al., 1999). The release of ferric iron from the porphyrin ring of heme during this reaction would not result in beneficial effects because this form of iron is known to catalyze oxidative reactions (Gutteridge et al., 1982). 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 Denner, 1999).

In our previous experiment, we had demonstrated that simvastatin induced a dose- and time-dependent upregulation of HO-1 protein expression in Neuro 2A cells (Hsieh et al., 2008). This upregulation of HO-1 was significantly associated with increased apoptosis in response to glucose deprivation, and the programed cell death could be significantly reduced by ZnPP and addition of the iron chelator desferrioxamine (DFO) (Hsieh et al., 2008). Therefore, we are interested in whether there is similar effect of statin-induced HO-1 on neuronal cells in response to different type of oxidative stress–like exposure to LPS, one situation that is common for those who sustain clinical infection. In addition, we are interested in whether there is different response between Neuro 2A cells and glial cells C6, which might be more responsive to the inflammation stimulation. However, all HO inhibitors that are currently available have numerous nonspecific actions that may complicate the interpretation of experimental results, including inhibition of nitric oxide synthase and guanylyl cyclase, modification of voltage-gated calcium currents (Grundemar and Ny, 1997; Meffert et al., 1994), and some inhibitors may also have a direct antioxidant effect that is unrelated to HO inhibition (Wagner and Dwyer, 2004). Therefore, additional inhibition of HO-1 with a more specific reagent like small-interfering RNA (siRNA) for HO-1 was performed to investigate the effect. Our results demonstrated that, when exposed to LPS, the increased HO-1 expression on the neuronal cells resulted in increased NF-κB activation as well as NADPH-dependent oxygen radical production, actions that were different from what happened in the endothelial cells. In addition, the viable cells were significantly decreased in 48 h in those cells that received simvastatin pretreatment plus LPS compared to those in control or exposed to simvastatin or LPS alone. Furthermore, blockade of HO-1 with ZnPP or siRNA for HO-1 and addition of DFO reverses the exaggerated oxidative response, which implied that iron production from HO-1 activity may play a role in the increased NF-κB activation and superoxides production by simvastatin-induced HO-1 in the neuronal cells exposed to LPS.

MATERIALS AND METHODS

Materials. Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin 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 Transduction Laboratories (Lexington, KY). Antibody of IκBα protein, IκBα phosphorylated at Ser32, and anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HO-1 siRNA and its transfection kit were obtained from Ambion (Austin, TX). All other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Cell culture. Neuro 2A mouse neuroblastoma and C6 rat glioma cells were obtained from Biresource 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. C6 cells were cultured in 82.5% Ham’s F10 medium supplemented with 2 mM l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 15% horse serum, and 2.5% FBS. Cells were kept in an atmosphere composed of 5% CO2/95% O2 at 37°C.

Experimental protocol. Neuro 2A and C6 cells were treated for the indicated time (3, 6, 12, and 24 h) in the presence of simvastatin (from 0.5 to 100 μM), HO-1 protein expression was determined in dose- and time-dependent fashion. HO-1 expression was determined in cells treated with 5 μg/ml LPS for 30 min in the presence or absence of simvastatin, HO-1 inhibitor ZnPP (10 μM), and HO-1 siRNA. The rodent HO-1 siRNA nucleotide sequence was as follows: 5’-GCCGGAAGUUCCAGUUUAC-3’. The control GAPDH siRNA nucleotide was obtained from Ambion. The transfection was performed with lipofectamine reagent according to the manufacturer’s instructions. IκBα phosphorylation, NF-κB subunit p65 nuclear translocation, and NADPH-dependent formation of reactive oxygen species (ROS) at the end of incubation were measured in neuronal cells exposed to LPS. The effect of an iron chelator DFO (30, 50, and 100 μM), a 50 mM CO donor [Ru(CO)₂(Cl)₂] (RuCO), and a 50 μM CO scavenger hemoglobin (Hb) on ROS formation was also evaluated.

HO-1 mRNA analysis. Using semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis, HO-1 mRNA analysis was performed in cells treated with simvastatin and/or LPS in the presence or absence of HO-1 siRNA. Total RNA, which was extracted from treated cells by applying 500 μl of RNAzol directly onto the cells with the addition of DNase digestion steps, was quantified 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. The primer set for detecting mouse HO-1 was as follows—sense primer: 5’-CGGGCGACGACAAAGTG-3’ and antisense primer: 5’-AGTGTTAGGACCCATCCGGAGA-3’. Quantification of HO-1 mRNA content was performed using a charge-coupled device camera (FluorChem 8900 imaging system; Alpha Innotech, San Leandro, CA). The intensity of each signal spot was transformed into digital data with autobackground subtraction during spot density analysis using AlphaEaseFC software.

Western blot analysis for HO-1 and NF-κB. Cytoplastic extracts were prepared from the Neuro 2A and C6 cells (2 × 10⁵ cells/ml) exposed to LPS and/or ZnPP in the indicated subgroups. The extracts were then resolved on 10% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, blocked with 5% skim milk in Tween-20 phosphate-buffered saline, and incubated with various primary antibodies (rabbit anti-HO-1, anti-IκBα, anti-pIκBα, anti-phosphoB, anti-p65, and...
mouse anti-β-actin). Anti-p65 antibody was used in the blotting for the nuclear-extracted protein. The blots were then incubated with HRP-conjugated secondary antibodies. The protein bands were quantified with FluorChem 8900 imaging system and the AlphaEaseFC software described above.

**Formation of ROS.** NADPH-dependent oxygen radical formation was measured by monitoring lucigenin-derived chemiluminescence at 37°C using the Berthold LB96V luminometer. Cells were cultured in six-well plates. After treatment with simvastatin for 24 h, cells were washed, trypsinized, and suspended in PBS and, subsequently, lucigenin (50 μM) and NADPH (100 μM) were added. LPS, ZnPP, and HO-1 siRNA were added to the suspended cells, respectively, as per the above protocol before the measurement of ROS formation. DFO was added at 5 h before treatment. RuCO and Hb were added 1 h before treatment, respectively. Chemiluminescence was measured in relative light units (RLU) every 5 min over a period of 60 min. Data shown represent means of peak values measured after 15 min as percentages of maximal light emission (%RLUmax) of NADPH-treated control cells.

**Cell viability assay.** Approximately 5000 Neuro 2A or C6 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 (50 μM for Neuro 2A and 80 μM for C6 cells) 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.

**Statistic analysis.** Results were expressed as mean ± SEM from at least three independent experiments. For comparison between multiple groups, statistical significance was tested by ANOVA and Student-Newman-Keuls tests post hoc or Student’s t-tests with SPSS statistical software (SPSS 13.0, Chicago, IL), assuming unequal variances. The p values <0.05 were considered statistically significant.

**FIG. 1.** Simvastatin induced neuronal HO-1 protein expression. (A) Concentration-dependent increases in HO-1 protein in Neuro 2A and C6 cells occurred following treatment with between 50 and 100 μmol/l simvastatin for 24 h. (B) Treatment of Neuro 2A with 50 μmol/l simvastatin and C6 cells with 80 μmol/l simvastatin resulted in a time-dependent upregulation of HO-1 protein expression. The induction of HO-1 by simvastatin was evident as early as 6 h, and the augmentation lasted for at least 24 h. Each bar represents the mean ± SD of three independently performed experiments; **p < 0.01; ***p < 0.001 versus control.
RESULTS

Simvastatin Induced HO-1 in Neuro 2A and C6 Cells

After treatment with various concentrations of simvastatin for 24 h, the Neuro 2A and C6 cells showed dose-dependent expression of HO-1 protein (Fig. 1A). Concentration-dependent increases in HO-1 protein occurred between 50 and 100 μM simvastatin in both cell lines (Fig. 1A). There was about a fourfold increase of HO-1 expression in Neuro 2A cells and about a sevenfold increase of HO-1 in C6 cells after 50 and 80 μM simvastatin, respectively. Treatment of Neuro 2A and C6 cells with simvastatin resulted in time-dependent upregulation of HO-1 protein expression (Fig. 1B). The induction of HO-1 by simvastatin was evident as early as 6 h, and the augmentation lasted for at least 24 h. The LPS could also be responsible for inducing significant HO-1 protein expression (Fig. 2A). However, significantly more HO-1 expression was noted in the cells treated with simvastatin plus LPS (Fig. 2A, lane 4) than in those treated with simvastatin or LPS alone (Fig. 2A, lanes 2 and 3). In addition, the induction of HO-1 was effectively inhibited by 10μM ZnPP (Fig. 2A).

Treatment with Simvastatin Plus LPS Increased NF-κB Activation

NF-κB activation was assessed by detecting IkBα phosphorylation and NF-κB subunit p65 nuclear translocation. Treatment with 5 μg/ml LPS for 30 min, but not with simvastatin, significantly increased IkBα phosphorylation both in the Neuro 2A and C6 cells (Fig. 2B, lanes 2 and 3). There was significantly more IkBα phosphorylation and p65 nuclear

FIG. 2. (A) Both simvastatin and LPS induced significant HO-1 protein expression; however, significantly more HO-1 expression was noted after treatment with simvastatin plus LPS. The induced HO-1 by simvastatin alone and by simvastatin plus LPS could be effectively inhibited by 10μM ZnPP. (B) Treatment with 5 μg/ml LPS alone for 30 min (lane 3) significantly increased IkBα phosphorylation and p65 nuclear translocation both in the Neuro 2A and C6 cells. Adding the LPS to the neuronal cell pretreated with simvastatin for 24 h induced significantly more IkBα phosphorylation and p65 nuclear translocation (lane 4). Inhibition of HO-1 with ZnPP abolished the exaggerated response of simvastatin plus LPS on NF-κB activation (lane 5). Each bar represents the mean ± SD of three independently performed experiments; **p < 0.01; ***p < 0.001 versus control or indicated; ###p < 0.001 versus treatment with simvastatin plus LPS.
translocation noted after treatment with simvastatin plus LPS than after exposure to LPS alone (Fig. 2B, lane 4 vs. lane 3). ZnPP effectively abolished the exaggerated response of NF-κB activation after simvastatin plus LPS treatment almost to the control level (Fig. 2B, lane 5).

Treatment with Simvastatin Plus LPS Increased ROS Formation

NADPH-dependent ROS formation was increased in Neuro 2A and C6 cells in a concentration-dependent fashion after treatment with simvastatin for 24 h (Fig. 3A). The increased ROS formation could be blocked in the presence of 10μM ZnPP in Neuro 2A cells but not in C6 cells (Fig. 3B, bar 4 vs. bar 2). When exposed to 5 μg/ml LPS for 30 min, the neuronal cells pretreated with simvastatin for 24 h showed significantly more increased ROS formation than those that had not received simvastatin treatment (Fig. 3B, bar 6 vs. bar 5). Moreover, the increased oxygen radical production was rescued in the presence of ZnPP (Fig. 3B, bar 7), which lowered ROS formation near to that of the control in Neuro 2A cells. Each bar represents the mean ± SD of three independently performed experiments; *p < 0.05; **p < 0.01; ***p < 0.001 versus control or indicated; ##p < 0.01; ###p < 0.001 versus indicated.

FIG. 3. (A) NADPH-dependent ROS formation was increased in a concentration-dependent fashion after treatment with simvastatin for 24 h. (B) Simvastatin-induced NADPH-dependent ROS formation was significantly attenuated in Neuro 2A cells but not in C6 cells by adding 10μM ZnPP. When exposed to LPS, both neuronal cells pretreated with simvastatin showed significantly increased ROS formation compared to those that had not received simvastatin treatment. The increased oxygen radical production was rescued in the presence of ZnPP, which lowered ROS formation near to that of the control in Neuro 2A cells. Each bar represents the mean ± SD of three independently performed experiments; *p < 0.05; **p < 0.01; ***p < 0.001 versus control or indicated; ##p < 0.01; ###p < 0.001 versus indicated.

HO-1 siRNA Inhibited ROS Formation

Induction of HO-1 by simvastatin plus LPS in Neuro 2A and C6 cells was inhibited effectively by HO-1 siRNA in a dose-dependent fashion for both the HO-1 transcripts and proteins (Fig. 4A). Transfection of HO-1 siRNA did not induce NADPH-dependent ROS formation in either neuronal cells (Fig. 4B, bar 6). The interference with the HO-1 transcripts by 3 and 10 nmol/l HO-1 siRNA in Neuro 2A cells and by 30 and 50 nmol/l HO-1 siRNA in C6 cells attenuated the increased ROS formation caused by treatment with simvastatin plus LPS (Fig. 4B, bars 4 and 5 vs. bar 2).

Iron Chelator DFO Reduced ROS Formation

In Neuro 2A cells, the addition of 50 and 100μM of DFO profoundly reduced NADPH-dependent oxidative stress after treatment with simvastatin alone (Fig. 5A, bars 7 and 8 vs. bar 2) or with simvastatin plus LPS (Fig. 5A, bars 11 and 12 vs. bar 9). DFO showed concentration-dependent blockade of aggravated NADPH-dependent ROS formation in Neuro 2A cells treated with simvastatin plus LPS, with ROS formation near to that of control cells when 100μM DFO was added (Fig. 5A, bar 12). Similarly, in C6 cells, NADPH-dependent ROS formation after treatment with simvastatin alone or with simvastatin plus LPS was rescued in the presence of DFO in concentrations ranging from 30 to 100μM (Fig. 5A). There was no significant effect on ROS formation under these conditions in the presence or absence of RuCO or Hb (Fig. 5B).
Effect of Simvastatin on Cell Growth

To evaluate the effects of simvastatin and LPS on Neuro 2A and C6 cell growth (the total number of cells/dish), cell viability was analyzed by MTT assay and showed that the viable cells were significantly decreased after 48 h by the treatment with simvastatin or LPS alone, when compared to the viable cells treated with cultured medium alone. Furthermore, the percentage of viable Neuro 2A and C6 cells was significantly decreased in those cells receiving simvastatin pretreatment plus LPS compared to the other three groups after 48 h (Fig. 6).

DISCUSSION

With the ability to cross the blood-brain barrier (Guillot et al., 1993) and potential neuroprotective properties, statins are promising candidates for the treatment of 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). In mouse models of cerebral ischemia, pretreatment with statins reduces infarct volume and provides protection from neurological deficits (Amin-Hanjani et al., 2001; Endres et al., 1998; Laufs et al., 2002). Among the statins investigated, simvastatin is the most effective for reducing oxidative stress and infarction volume after middle cerebral artery ischemia and reperfusion injury (Hayashi et al., 2005). In cultured neurons, statins have also been shown to elicit resistance to N-methyl-D-aspartic acid-induced excitotoxic death (Zacco et al., 2003). However, although the neuroprotective effects of different statins have been demonstrated in other experimental settings, in a rat model of autoimmune optic neuritis, simvastatin was unable to demonstrate an increase in either retinal ganglion cell survival or an improvement of visual function. In contrast, it has also been reported that treatment of astrocytes with atorvastatin and simvastatin induced 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.
These controversial reports make the further investigation of the effect of statins on neuronal cells mandatory before their use for such purposes. The cholesterol-independent cytoprotective effects of statins have been attributed to induction of HO-1 (Grosser et al., 2004a,b; Lee et al., 2004), elevations in eNOS (Laufs et al., 2002; Loboda et al., 2006) and Bcl-2 (Franke et al., 2007), expression of tissue-type plasminogen activator or heat shock protein (Kretz et al., 2006; Wierzbicki et al., 2003), and the inhibition of geranylgeranylation (Bi et al., 2004). Among these crucial protective and anti-inflammatory genes, HO-1 is particularly involved in the protective mechanism against vascular injury. The expression of HO-1 acts against oxidative stress in microglia, astrocytes, and neurons following distinct experimental models of pathological alterations to the brain such as subarachnoidal hemorrhage, ischemia, and traumatic brain injury and in human neurodegenerative diseases (Beschorner et al., 2000). HO-1 induction in the brain reduces stroke-related ischemic injury and might therefore contribute to the main neuroprotective actions of statins (Maines, 2002). In the present study, our data demonstrated that simvastatin induced concentration-dependent increases in HO-1 protein expression in neuronal cells. However, when exposed to LPS, the upregulation of HO-1 after simvastatin treatment was significantly associated with increased, not decreased, NF-κB activation and NADPH-dependent oxygen radical production, phenomena which are different from that the statin provided to endothelial cells (Grosser et al., 2004a,b) and to vessels (Lee et al., 2004). Inhibition of the induced HO-1 by either ZnPP or HO-1 siRNA reduced the increased ROS production, implying that HO-1 expression was involved in the increased production of oxygen radicals.

In this experiment, HO-1 expression was induced in both neuronal cell lines by high concentrations of simvastatin, i.e., 50–100μM, which are much higher than their pharmacological concentrations in lowering cholesterol. Loboda et al. (2006)
has suggested that the protective effect of statins is mediated by enhancement of eNOS and does not involve HO-1 expression (Loboda et al., 2006). Their study demonstrated that atorvastatin, at a pharmacologically relevant concentration (0.1 mM), enhanced the expression of eNOS, but not HO-1, in human microvascular endothelial cells (HMEC-1) and prevented the hypoxia-induced decrease in eNOS expression. Enhanced cytotoxicity of atorvastatin on HMEC-1 has been noted already at the 3 mM concentration, and this effect is aggravated at 10 mM (Loboda et al., 2006). In two other studies, Grosser et al. (2004b) demonstrated that the induction of HO-1 expression by lovastatin and simvastatin (Grosser et al., 2004b) as well as by rosuvastatin (Grosser et al., 2004a) at 100–300 μM concentrations had cytoprotective actions in ECV304 and EA.hy 926 endothelial cells. Simvastatin has also been shown to attenuate LPS-induced TNF-α expression in cardiomyocytes via inhibition of activation of NADPH oxidase and subsequent ROS generation (Shang et al., 2006) and to inhibit cytokine expression in rat and human myocardium (Wallace et al., 2005; Zhang et al., 2005). However, the response of neuronal cells to statins might be quite different to that of endothelial cells and cardiomyocytes, thus raising some concerns about interpreting the physiological relevance of experiments in vitro or in vivo on different cell types and with different concentrations of statins (Brown et al., 2000). Whether the protective mechanism in endothelial cells but not in the neuronal cells is attributed to the upregulation of eNOS, which is easily induced in endothelial cells, or other proteins require further experiment to validate.

Cells with the highest levels of HO-1 expression had the highest reactive iron content, which is the likely candidate for HO-1–mediated toxicity. Iron toxicity is largely based on Fenton chemistry where iron reacts with reactive oxygen intermediates, including hydrogen peroxide (H₂O₂) and the superoxide anion (O₂⁻), to produce highly reactive free radical species (Gaasch et al., 2007). Exaggerated release of ferric iron (Fe³⁺) from the porphyrin ring of heme by HO-1 would result in prominent oxidative reactions and be associated with increased generation of oxygen radicals (Suttner and Dennery, 1999). Notably, the quantity of free radicals produced is directly proportional to the amount of reactive iron (Nunez-Millacura et al., 2002). In fact, many studies have shown that reactive iron is associated with increased oxidative stress in human disease. In conditions such as subarachnoid or intracerebral hemorrhage, the heme within Hb, the iron-containing component of erythrocytes, is cleaved by heme oxygenase (HO-1 and HO-2) to produce biliverdin, carbon monoxide, and free ferrous iron or can be degraded by hydrogen peroxide to release free iron and has been demonstrated to be highly neurotoxic in vitro (Levy et al., 2002). It was shown in the present study that both the inhibition of HO-1 expression and chelation of cellular iron with DFO significantly reduced the formation of ROS, implying that active iron might be involved in increased oxidative reactions after treatment with simvastatin plus LPS. However, because the regulation of iron transport and storage by iron binding protein in iron overload conditions is complicated in the body (Gaasch et al., 2007), whether there are similar effects in vivo require further investigation in animal model. Furthermore, although Suttner and Dennery (1999) reported that cytoprotection was associated with less than fivefold HO-1 expression and significant oxygen cytotoxicity with greater than 15-fold HO-1 expression in hamster fibroblasts (HA-1), we did not find such a duality of effects to HO-1 overexpression in this study. Initial fourfold and sevenfold HO-1 expression in Neuro 2A and C6 cells, respectively, from high concentration simvastatin therapy led to an increased neuronal NF-κB activation and oxidative stress.

![Figure 6](image_url)  
**FIG. 6.** Cell viability was determined by the MTT assay in the presence and absence of simvastatin treatment and LPS. Results are expressed as the fold of viable cells relative to untreated cells in 0, 24, and 48 h.
response to LPS. In addition, the viable cells detected from the MTT assay were significantly decreased in those cells that received simvastatin plus LPS treatment compared to those in control or exposed to simvastatin or LPS alone. It has also been reported that excessive HO-1 activity may exacerbate brain damage in a host of degenerative and inflammatory neurological conditions (Schipper et al., 1999; Lu and Ong, 2001). Different types of damage that affect the striatal neurons or terminals induce early overexpression of HO-1 in striatal glia, and it has been suggested that the presence of HO-1 immunoreactivity may be used as a useful neuroanatomical marker for early stage striatal damage (Munoz et al., 2005). Therefore, novel therapeutic approaches that induce overexpression of HO-1 must be considered with caution.

In summary, simvastatin-induced HO-1 was associated with increased NF-kB activation and NADPH-dependent oxygen radical production in cultured neuronal cells exposed to LPS. Inhibition of HO-1 or chelation of iron was able to reduce oxygen radical production, implying that downstream iron release from HO-1 activity may play an important role in the exaggerated response.

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REFERENCES


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