Effects of Simvastatin on the Expression of Heme Oxygenase-1 in Human RPE Cells

Kyoung Jin Kim, Kyong Sil Kim, Na Rae Kim, and Hee Seung Chin

Purpose. Chronic oxidative stress can lead to the impairment of RPE cells, indicating it to be a risk factor for AMD. The cholesterol-independent, pleiotropic effects of statins have protective effects on several cell types via unknown mechanisms. This study examined the role of heme oxygenase-1 (HO-1) as a target and potential mediator of statins in cultured human RPE cells.

Methods. The RPE cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. After 24 hours incubation, RT-PCR and Western blot was performed to measure the levels of HO-1 mRNA and protein expression, respectively, in RPE cells. Intracellular reactive oxygen species (ROS) production was measured using a fluorescence-activated cell sorter.

Results. In cultured human RPE cells, simvastatin showed no toxicity up to 10 μM. Simvastatin increased the HO-1 mRNA and protein levels in a concentration-dependent manner up to 10 μM. HO-1 protein induction by simvastatin was unaffected by mevalonate or N-nitro-L-arginine methyl ester, showing that the isoprenoid- and NO-dependent pathways are not involved. Simvastatin-dependent HO-1 protein induction was reduced significantly by pharmacological inhibition of the phosphotidylinositol-3-kinase (PI3K)/Akt pathways. The simvastatin-induced inhibition of free radical formation was recovered by the presence of an HO inhibitor, zinc protoporphyrin.

Conclusions. These results demonstrate that HO-1 is a target site and an antioxidant mediator of simvastatin in human RPE cells. Simvastatin-dependent upregulation of HO-1 is mainly via PI3K/Akt-dependent signaling pathways. Simvastatin may have some clinical benefits in preventing retinal diseases associated with oxidative stress, such as AMD. (*Invest Ophthalmol Vis Sci.* 2012;53:6456–6464) DOI:10.1167/iovs.12-9658

From the Department of Ophthalmology and Inha Vision Science Laboratory, Inha University School of Medicine, Incheon, Republic of Korea.

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Corresponding author: Hee Seung Chin, Department of Ophthalmology and Inha Vision Science Laboratory, Inha University Hospital, 7-206 Shinheung-dong, Jung-gu, Incheon, Republic of Korea; hschin@inha.ac.kr.

AMD is the leading cause of blindness in the United States. In the 2005–2008 National Health and Nutrition Examination Survey, the prevalence of any type of AMD in the U.S. population aged 40 years and older was 6.5%, and the estimated prevalence of late AMD was 0.8%.[1] The visual impairment in AMD is due to the destruction of photoreceptors in the macula and the degeneration of the RPE layer and Bruch's membrane.[2]

The RPE layer is a single layer between the retina and choriocapillaris, acting as a blood-retinal barrier.[3] The retina is a highly oxygen-consuming tissue that functions under high oxygen tension. Hence, RPE cells are always exposed to oxidative stress. This chronic oxidative stress can lead to the impairment and death of RPE cells, suggesting it to be a risk factor of various retinal diseases.[4,5]

Producing stress proteins under oxidative stress is a universal protective response in cells.[6] RPE cells also produce some defense enzymes against oxidative stress.[7] One of them is heme oxygenase (HO), a heat-shock protein. HO catalyzes the first rate-limiting step in the heme catabolism. HO degrades heme into equimolar amounts of carbon monoxide, free ferrous iron, and biliverdin.[8] HO-1, which is the inducible form of HO, is distributed evenly in human organs and can be induced by a range of internal or external stimuli including oxidative stress.[9–13] Cytoprotective actions of HO-1 have been documented in many tissues, including the heart, kidney, vascular smooth muscle cells, endothelial cells, and neuronal cells.[14]

HMG-CoA reductase inhibitors, also known as statins, are lipid-lowering agents used widely in medicine. Statins can reduce cardiovascular mortality and prevent the progression of atherosclerosis in patients with coronary artery disease.[15] In addition, statins have anti-inflammatory, anti-oxidative, and antiangiogenic effects, all of which are related to the pathogenesis of AMD.[16–18] These pleiotropic, cholesterol-independent effects of statins include a decrease in proinflammatory signaling, oxygen radical formation, and induction of the HO-1 protein. This study examined whether the anti-oxidative effect of simvastatin occurs mainly through HO-1.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Rabbit polyclonal antibodies specific to HO-1, ERK, p38 MAPK, PI3K/Akt, PKG, JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies specific to phosphorylated ERK, p38 MAPK, PI3K/Akt, PKG and JNK were purchased from Cell Signaling Technology (Beverly, MA). Mevalonate, N-nitro-l-arginine methyl ester, PD98059, SB203580, LY294002, KT5823, SP600125, SB202190, Rp-8-Br-PET-cGMPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit were obtained from Sigma-Aldrich (St. Louis, MO). Simvastatin was supplied by CJ Pharma (Seoul,

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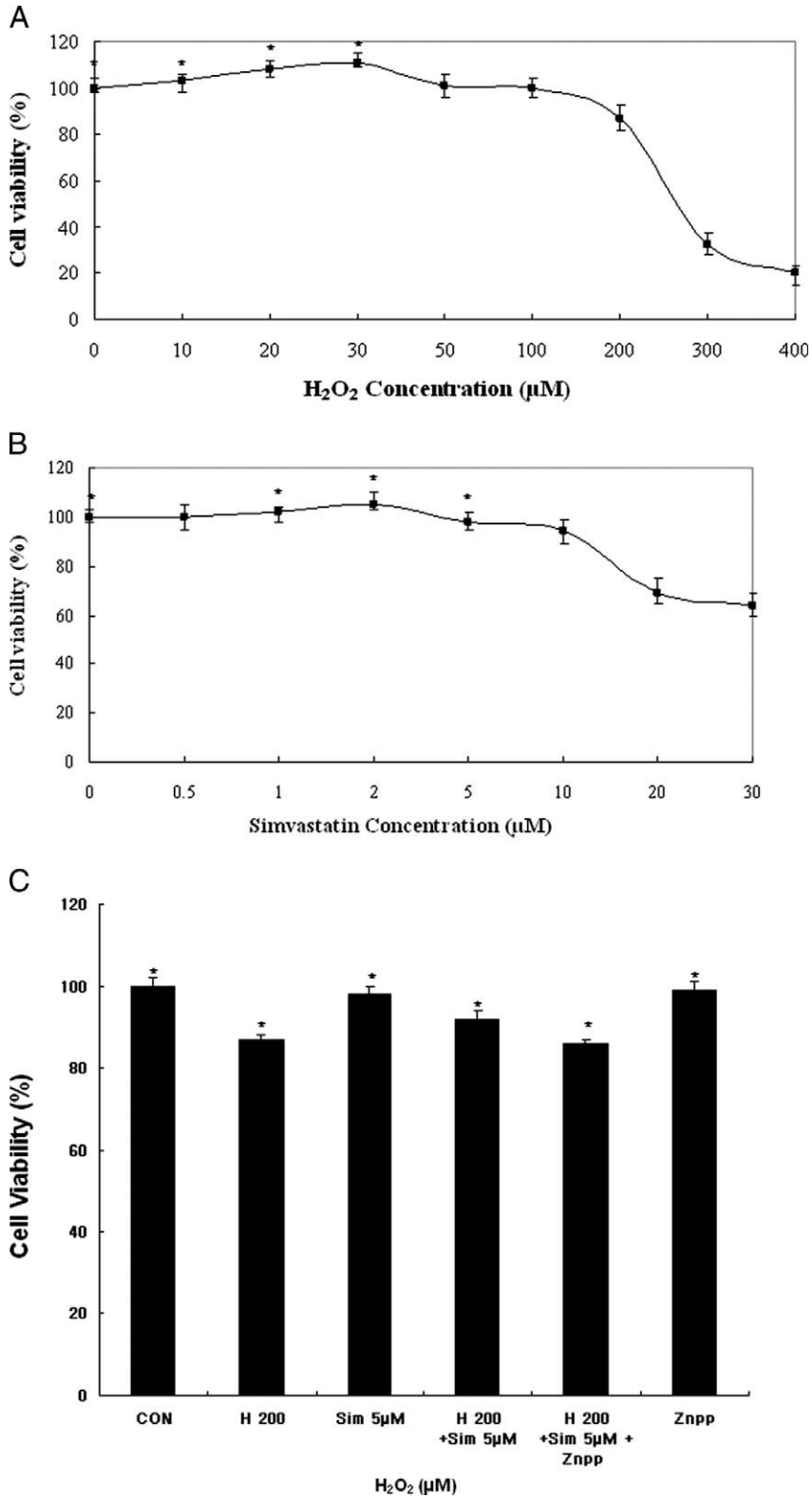

Figure 1. The viability of ARPE-19 cells upon exposure to H_2O_2 or simvastatin. (**A**) The cell viability was conserved until 200 μM H_2O_2. On the other hand, the cell viability showed a progressive decrease with increasing H_2O_2 concentrations at more than 200 μM. (**B**) The cell viability was conserved until 10 μM of simvastatin but cell survival showed a progressive decrease with increasing simvastatin concentrations >10 μM and toxicity was observed at 20 μM simvastatin. (**C**) The cell viability slightly increased when 5 μM simvastatin was added in 200 μM H_2O_2 pretreated cell. And addition of ZnPP was abolishing that protection. ZnPP alone had no significant effect on cell viability. The cell viability was determined using a MTT assay. The experiment was performed five times independently. $^*P < 0.05$ versus the control.

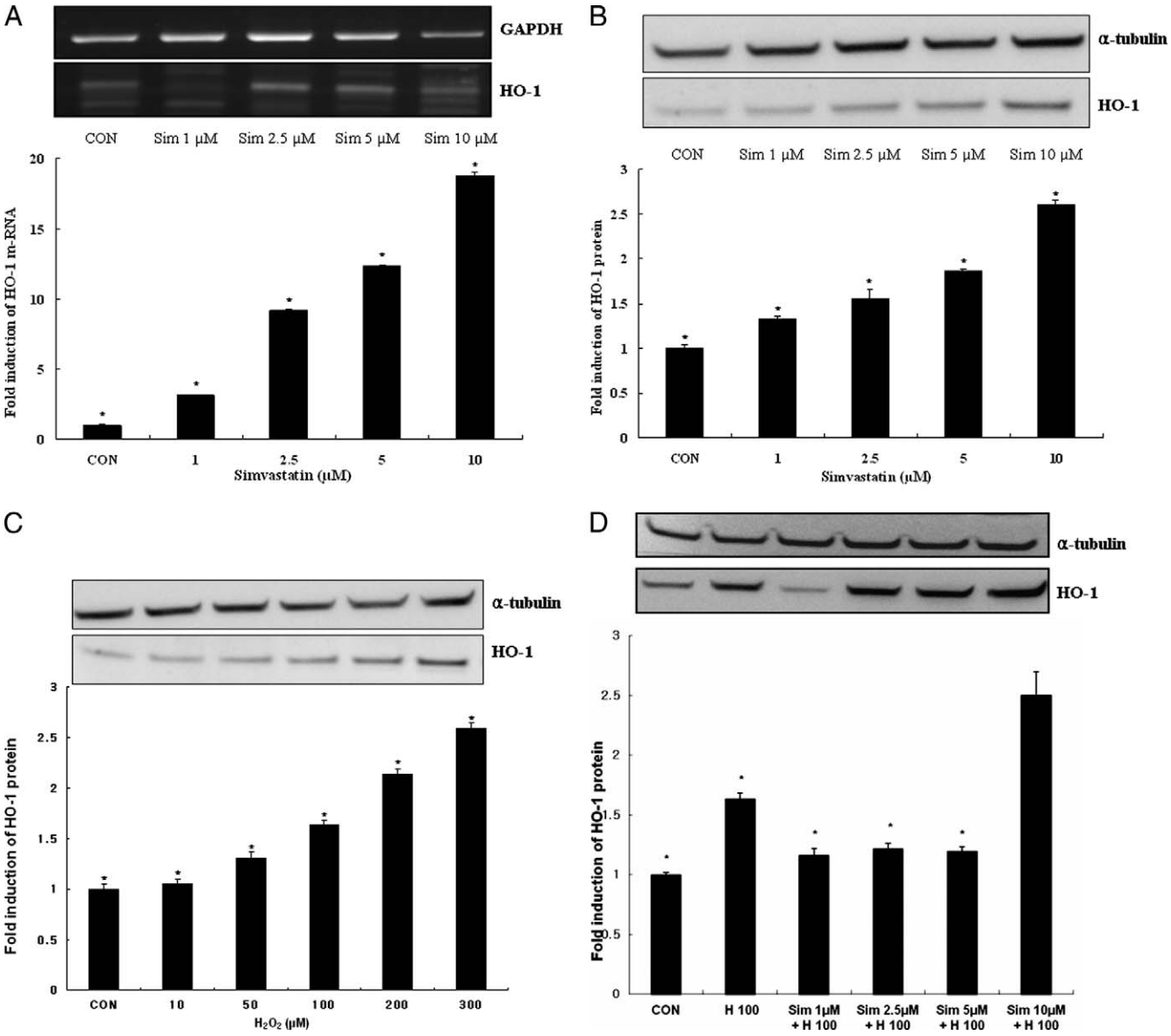

Figure 2. Effect of simvastatin (Sim) on HO-1 expression in ARPE-19 cells in a concentration-dependent manner. Human RPE cells were cultured with various simvastatin concentrations for 24 hours. (**A**) Expression of HO-1 mRNA increased with increasing simvastatin concentration. Expression of HO-1 mRNA was normalized by GAPDH (housekeeping gene). (**B**) The expression of HO-1 protein increased with increasing simvastatin concentration. (**C**) The expression of HO-1 protein increased with increasing H_2O_2 concentration, in a concentration-dependent manner. (**D**) HO-1 protein production was decreased after addition of 1, 2.5, 5 μM simvastatin in 100 μM H_2O_2 pretreated cells. However, after being treated with 10 μM simvastatin, HO-1 protein production has increased (not statistically significant). Expression of the HO-1 protein was normalized by α-tubulin. The data is reported as the mean ± SD of five experiments performed independently. $^*P < 0.05$ versus the control.

Republic of Korea). H_2DCFH-DA was obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich and Roche (Penzberg, Germany).

Cell Culture

Human RPE cells (ARPE-19; ATCC No.CRL-2302) were cultured in a humidified incubator at 37°C in an atmosphere containing 95% air and 5% CO_2. The cells were grown in a 1:1 mixture of basal DMEM and Ham's F12 medium (DMEM/F-12) containing 10% FBS and 1% antibiotic-antimycotic. The culture medium was changed every 3 days. The cells between passages 5 and 6 were used for the experiments. The ARPE cells were grown in plates until they reached 80% ~ 90% confluence. Cells were washed with PBS and starved for 6 hours, prior to processing the experiments.

Cell Viability Test

The effects of simvastatin and H_2O_2 on RPE cell growth were examined by determining the cell viability using an MTT assay. Human RPE cells were diluted to 5×10^3 cell/mL and seeded onto 96-well microplates (Falcon, Lincoln Park, NJ) at 90 μL each. Simvastatin was dissolved in dimethyl sulfoxide (DMSO) before being added to the medium. After growing for 18 hours, they were challenged with PBS as the control media, H_2O_2 (0–400 μM) and simvastatin (0–30 μM), each for 24 hours. Subsequently, 10 μL of an MTT solution (5 mg/mL) was added and cultured for an additional 4 hours at 37°C. After washing out the culture media, 150 μL of DMSO was added to each well for 10 minutes and the insoluble MTT formazan crystals were dissolved. The absorbance was measured at 540 nm on a microplate reader (Bio-

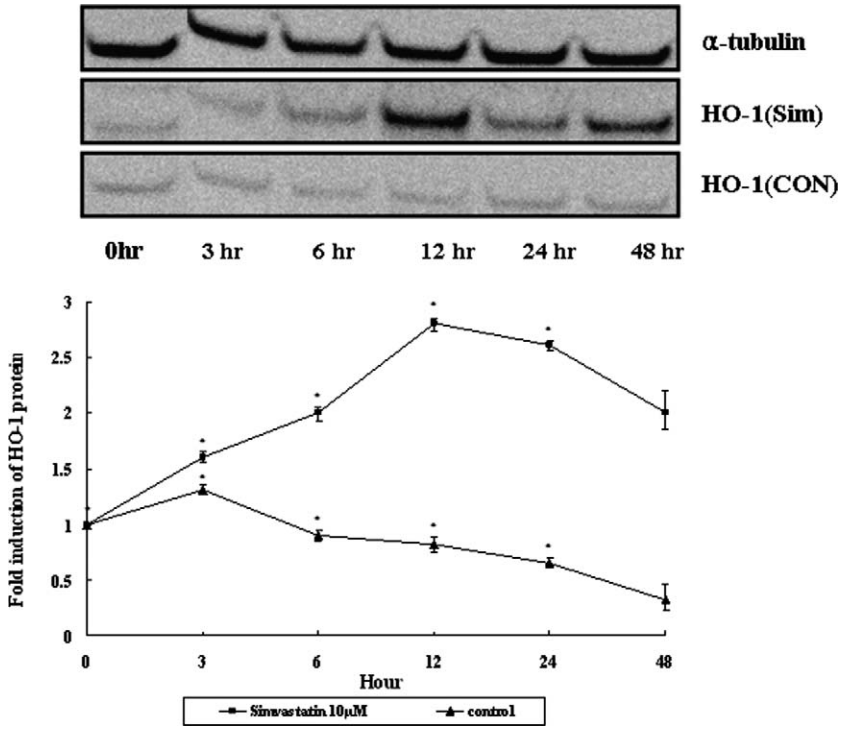

Figure 3. Effect of simvastatin on HO-1 expression in ARPE-19 cells in a time-dependent manner. The RPE cells were challenged with 10 μM simvastatin for different times, resulting in the time-dependent upregulation of HO-1. The induction of the HO-1 protein by simvastatin was evident as early as 3 hours. The highest level was expressed at 12 hours with the effect lasting for at least 48 hours. The data is reported as the mean ± SD of five experiments performed independently. $^*P < 0.05$ versus control.

Tek, Cambridge, MA). The optical density of the formazan formed in the control cells was considered to be 100% cell viability.

HO-1 mRNA Analysis

Subconfluent RPE cells were diluted to 1×10^6 cell/mL and incubated for 24 hours in 6-well plates. After washing out the culture media with PBS twice, serum-free media was applied. Simvastatin was then added at different concentrations (0–10 μM) and incubated for 24 hours. The total RNA was isolated using a Total RNA Purification kit (Invitrogen). The isolated RNA was quantified and 1 μg of the total RNA and 100 pmole of oligo dT were added to the RT Premix to make 20 μL; cDNA. Preformed cDNA, HO-1 primer, and glycerol-3-phosphate dehydrogenase (GAPDH) primer were mixed to a PCR Premix and PCR was performed. Subsequently, 10 μL of each amplificate was assessed by 1.5% agarose gel electrophoresis. Primer sequences used were: HO-1 forward, 5′-GTCCGCAACCCGACAGCA-3′; HO-1 reverse, 5′-TCCTCCAGGGCCACATAGATG-3.′ Quantification of the HO-1 mRNA content was performed using computer-assisted videodensitometry (Eagle Eye II-system; Stratagene, La Jolla, CA).

HO-1 Protein Analysis

After 24 hours incubation with the control medium or simvastatin (0–10 μM), subconfluent RPE cells were washed and extracted. The extracts were then resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane and immunoblotted using an immunoblotting device (iBlot; Invitrogen) with the polyclonal antibody to rabbit HO-1. The blots were then incubated with the horseradish peroxidase-conjugated secondary antibodies. The autoradiograms were scanned by densitometry and the results were normalized to the corresponding α-tubulin signal.[19]

Formation of ROS

The ARPE-19 cells were pretreated with 10 μM simvastatin or 100 μM hydroperoxide. The medium was removed and labeled with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) diluted in HBSS. In this process, H_2DCFH-DA is converted to dichlorofluorescin (DCFH) in the cell, and is further converted to dichlorofluorescein (DCF) in the presence of reactive oxygen species. The cells were incubated for 30 minutes at 37°C, the dye was removed, and the cells were then washed with HBSS. The level of intracellular ROS production was measured and quantified using a fluorescence activated cell sorter (FACS; Invitrogen).[20] HO inhibitor, zinc protoporphyrin (ZnPP), was added 15 minutes before the addition of simvastatin.

Statistical Analysis

The results are expressed as the mean ± SD of at least five experiments performed independently. Statistical analysis was carried out using statistical software (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL). A Kruskal–Wallis test was used to compare the control and experimental groups. A P value < 0.05 was considered significant.

Results

Cell Viability Test

An MTT assay was performed with various concentrations to determine the optimal level of H_2O_2 and simvastatin. The cell viability after 24 hours incubation with 50, 100, and 200 μM H_2O_2 was 98.3 ± 6.8%, 97.3 ± 6.3%, and 87 ± 5.5%, respectively. The cell viability decreased gradually with increasing H_2O_2 concentration (Fig. 1A). As shown in Fig. 1B, simvastatin showed no toxicity to human RPE cells up to

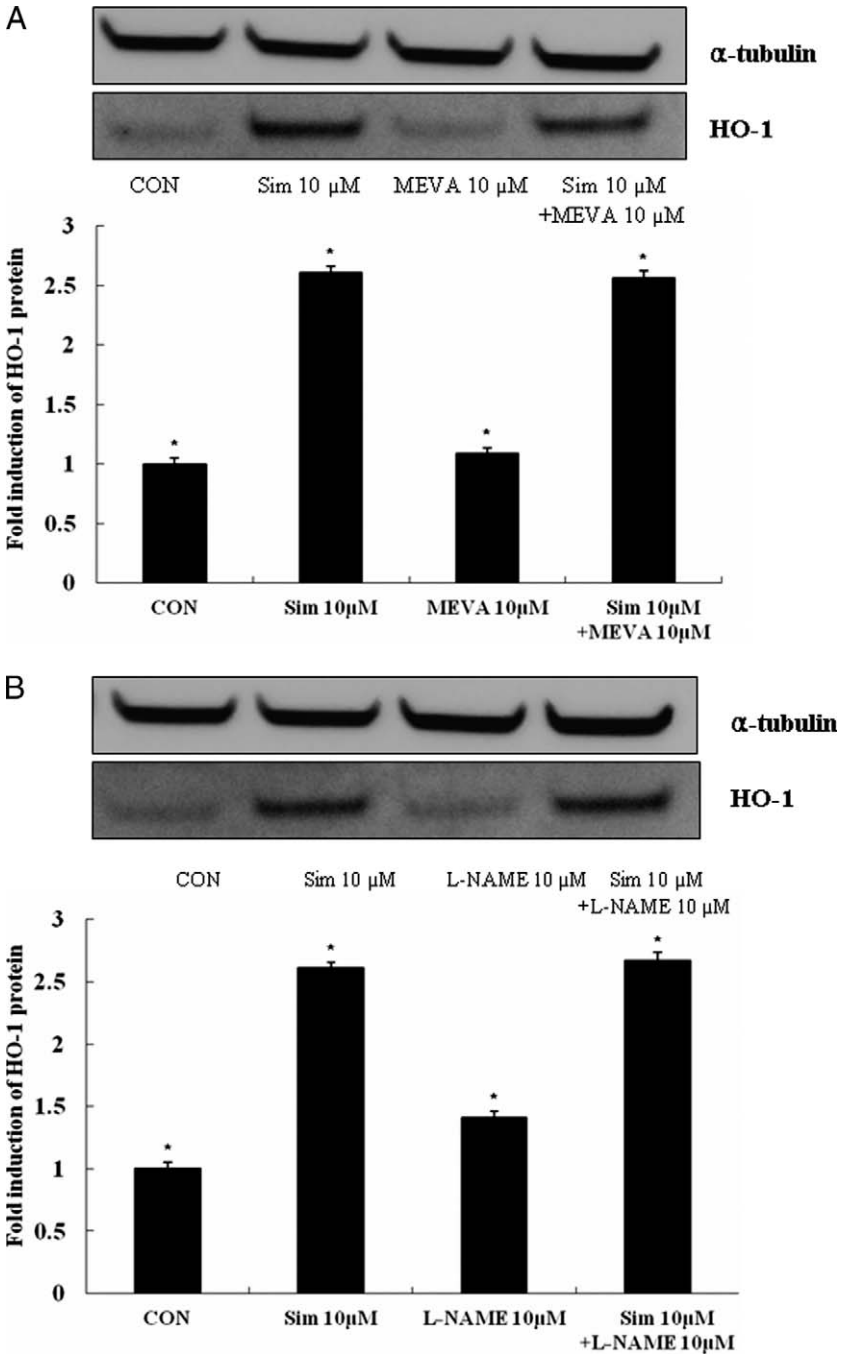

Figure 4. Effect of the HMG-CoA product and the NO synthase inhibitor on the simvastatin-dependent HO-1 protein induction in human RPE cells. (**A**) Effect of the HMG-CoA product, mevalonate (MEVA), on simvastatin (Sim)-induced HO-1 protein expression in RPE cells. (**B**) Effect of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), on simvastatin-induced HO-1 protein expression in RPE cells. HO-1 protein induction by simvastatin was unaffected by mevalonate and L-NAME. The blot shown is representative of five experiments with similar results. The data is reported as the mean ± SD of five experiments performed independently. $^*P < 0.05$ versus the control.

10 μM. The cell viability decreased at a much slower rate and was maintained at >94.1 ± 6.3% until the simvastatin concentration reached 10 μM. After 24 hours incubation with 200 μM H_2O_2, the cell viability have decreased than control (87 ± 5.5%), however, the cell viability slightly increased when 5 μM simvastatin was added (98 ± 6.1%). And addition of ZnPP abolished that protection (86 ± 3.5%). ZnPP alone had no significant effect on cell viability (Fig. 1C).

Simvastatin-Induced HO-1 Expression in RPE Cell

After treatment with various simvastatin concentrations for 24 hours, reverse transcription polymerase chain reaction (RT-PCR) and Western blot were performed to detect the HO-1 mRNA and protein, respectively. The level of HO-1 mRNA expression increased with increasing simvastatin concentration up to 10 μM (Fig. 2A). A concentration-dependent increase in HO-1 induction was also observed at the protein level (Fig.

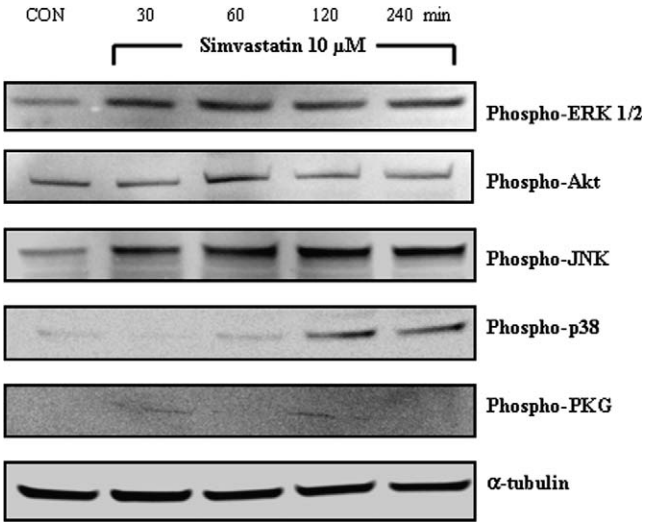

Figure 5. Effect of simvastatin on ERK, p38 MAPK, Akt, PKG, and JNK phosphorylation in ARPE-19 cells. The cells were incubated with 10 μM simvastatin for the times indicated (15, 30, 60, 120, 240 minutes). Western blot analysis was performed and the phosphorylation of the respective kinases was detected using phospho-specific antibodies against ERK, p38 MAPK, Akt, PKG, and JNK. ERK, Akt, JNK, and p38 MAPK were phosphorylated in a time-dependent manner. In contrast, PKG was very slightly phosphorylated. Equal loading was verified using the antibodies against total ERK, p38 MAPK, Akt, PKG, and JNK. Representative autoradiograms of at least five independent experiments with similar results are shown.

2B). Also, the expression of HO-1 increased with increasing H_2O_2 concentration, in a concentration-dependent manner (Fig. 2C). In 100 μM H_2O_2 treated cells, HO-1 protein production was decreased after the addition of 1, 2.5, 5 μM simvastatin. However, after being treated with 10 μM simvastatin, HO-1 protein production increased (Fig. 2D). RPE cells challenged with 10 μM simvastatin for different times showed a time-dependent upregulation of HO-1 protein expression for up to 12 hours (Fig. 3).

Signaling Pathways of Simvastatin Induced HO-1 Expression

The HMG-CoA product, mevalonate, was used to determine if HO-1 induction by simvastatin was mediated via the isoprenoid pathway. The simvastatin-induced HO-1 protein levels were unaffected by mevalonate (Fig. 4A). L-NAME, an NO synthase inhibitor, also had no effect on HO-1 protein expression (Fig. 4B). Therefore, RPE cells were incubated with simvastatin for various times up to 4 hours to determine its effect on the activation of protein kinases, such as ERK, p38 MAPK, PI3K/Akt, PKG, and JNK. The results showed that ERK, Akt, JNK, and p38 MAPK were phosphorylated in a time-dependent manner (Fig. 5). However, PKG was very slightly phosphorylated. The effects of inhibitors of the signaling intermediates on HO-1 protein expression were examined to determine the signal transduction pathway of HO-1 expression in response to simvastatin. ARPE-19 cells were preincubated for 30 minutes with the chemical inhibitors of protein kinases before incubation with simvastatin. The p38 MAPK inhibitor (SB203580, SB202190) and PKG inhibitor (KT5823, Rp-8-Br-PET-cGMPS) had no effect on the simvastatin-dependent HO-1 induction in RPE cells. The ERK inhibitor (PD98059) and JNK

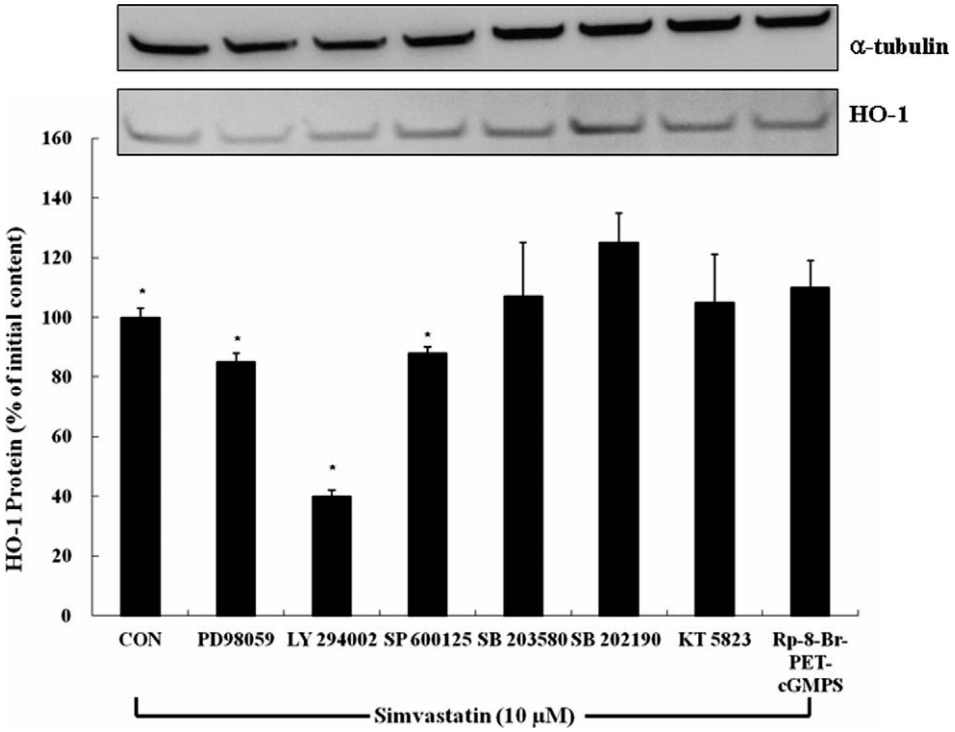

Figure 6. Effect of pharmacological protein kinase inhibitors on the simvastatin-dependent HO-1 protein induction in human RPE cells. The cells were preincubated separately with 10 μM PD98059, LY294002, SP600125, SB203580, SB202190, KT5823, and Rp-8-Br-PET-cGMPS for 30 minutes before adding simvastatin. Simvastatin-dependent HO-1 protein induction was reduced significantly by pharmacological inhibition of the PI3K/Akt pathways. Western blot analysis was performed. The data is reported as the mean ± SD of five experiments performed independently. $^*P < 0.05$ versus the control.

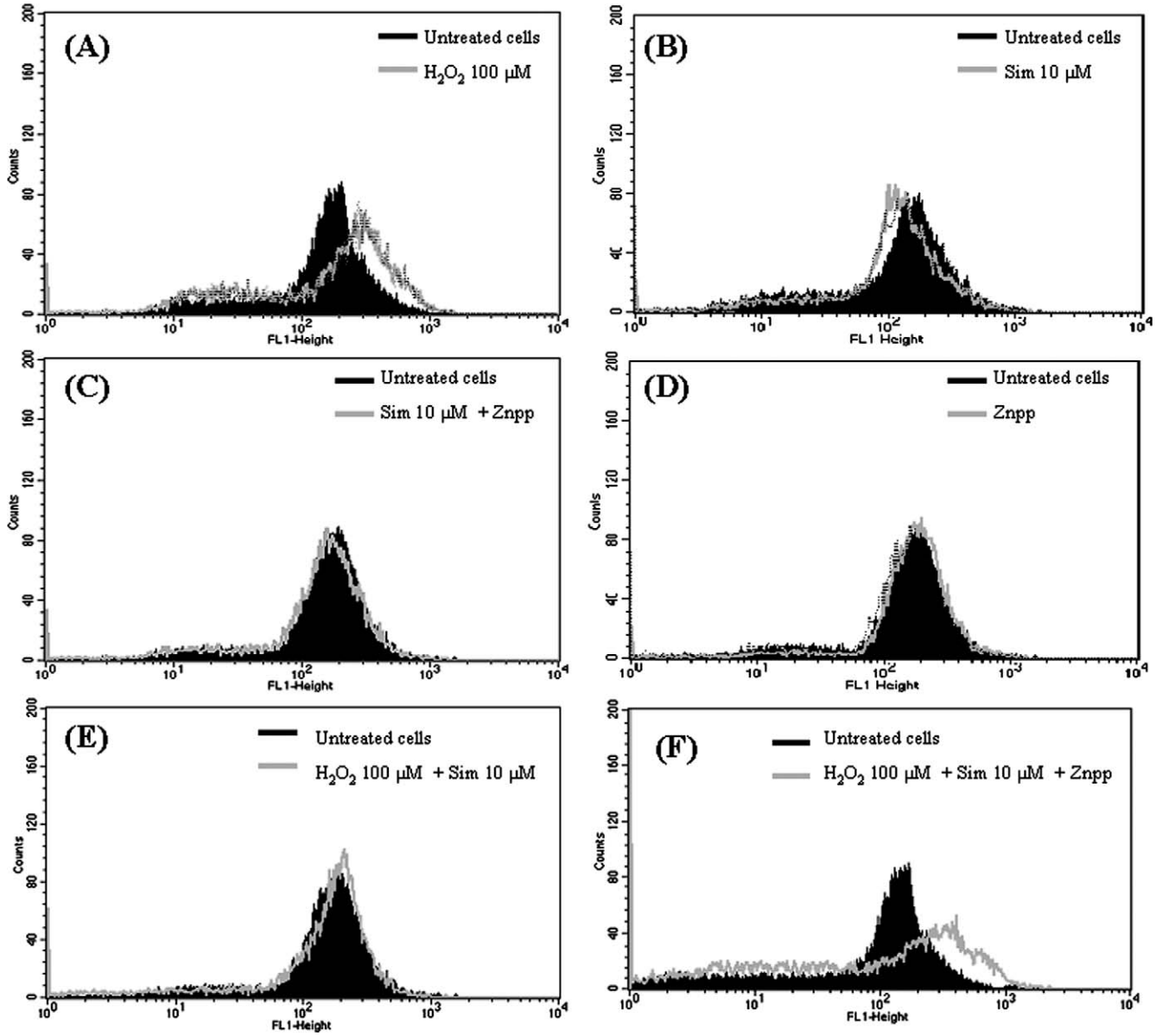

Figure 7. Effect of simvastatin on ROS formation in human RPE cells. (**A**) ROS formation was increased in human RPE cells after a pretreatment with H_2O_2. (**B**) ROS formation was inhibited in human RPE cells after a pretreatment with simvastatin. (**C**) Simvastatin-induced blockade of free radical formation was reversed in the presence of a HO inhibitor, ZnPP. (**D**) ZnPP alone had no significant effect on ROS formation. (**E**) Increased ROS formation was recovered in H_2O_2-treated human RPE cells after being pretreated with simvastatin. (**F**) This effect was abolished by HO-1 inhibitor, ZnPP. The data is reported as the mean ± SD of five experiments performed independently.

inhibitor (SP600125) had a minor effect on the simvastatin-dependent HO-1 induction. On the other hand, inhibitors of PI3K/Akt (LY294002) reduced simvastatin-dependent HO-1 stimulation significantly (Fig. 6). Protein kinase inhibitors itself did not increase the HO-1 protein level in human RPE cells.

Antioxidative Effects of Simvastatin

To quantify the degree of cellular oxidative stress, FACS was used to measure the level of ROS. ROS formation was higher in the RPE cells treated with 100 μM H_2O_2 (Fig. 7A). ROS formation was rescued in the RPE cells after a pretreatment with 10 μM simvastatin (Fig. 7B). The role of HO-1 in the simvastatin-induced antioxidative effect was examined using ZnPP, an HO-1 inhibitor.[19] The simvastatin-induced blockade of free radical formation was recovered in the presence of ZnPP (Fig. 7C). Increased ROS formation was recovered in H_2O_2-treated human RPE cells after a treated with simvastatin (Fig. 7E). And this effect was abolished by HO-1 inhibitor, ZnPP (Fig. 7F). ROS formation was increased in human RPE cells after a pretreatment with H_2O_2 and not changed with ZnPP addition (data not shown). ZnPP alone had no significant effect on ROS formation (Fig. 7D).

Discussion

Statins modify the lipid profile of patients and decrease their risk of coronary heart disease. They also prevent stroke and possibly Alzheimer disease. In recent years, several studies have suggested that statins also have protective effects in AMD because of their antioxidant activity.[21,22] The continued intake

of statins appears to be at least equal to the prophylactic effect of multivitamin and antioxidant supplements, as reported in the Blue Mountain Eye Study.[22]

Statins lead to promoter activation of the antioxidative defense protein, HO-1, which might explain their pleiotropic antioxidative, anti-inflammatory actions.[23] HO-1 is an inducible enzyme that catalyzes the degradation of heme to biliverdin with the concomitant release of iron and carbon monoxide. Over the past few years, many 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, as well as via the anti-inflammatory action of carbon monoxide.[24–26]

Several studies have reported that statins induce HO-1 in many cell types. Simvastatin has anti-inflammatory and antiproliferative effects in rat aortic vascular smooth muscle cells both in vitro and in vivo, largely through the induction of HO-1.[27] Simvastatin also increased the expression of HO-1 in human endothelial cells. The simvastatin-dependent upregulation of endothelial HO-1 is regulated mainly by the stabilization of HO-1 mRNA via a PI3K/Akt-dependent signaling pathway.[28] On the other hand, in neuronal cells, simvastatin-induced HO-1 leads to increased NF-κB activation and superoxide production when exposed to LPS.[14]

This study observed the effects of simvastatin on HO-1 expression in human RPE cells. Simvastatin was used in this study due to its widespread use in laboratory settings and it is a synthetic analogue of the naturally occurring statin, lovastatin. The MTT assay was performed to determine the toxicity of simvastatin to human RPE cells. In the case of simvastatin, the RPE cell viability was maintained up to 10 μM (Fig. 1B), suggesting that simvastatin is relatively nontoxic to RPE cells at lower concentrations. And addition of simvastatin was protective and ZnPP was abolishing that protection (Fig. 1C). These results were similar to previous reports.[20,29] Simvastatin produced concentration-dependent increases in HO-1 mRNA and protein expression up to 10 μM (Figs. 2A, 2B). HO-1 protein production was decreased after addition of simvastatin in 100 μM H_2O_2-treated cells. These effects were also observed at micromolar concentrations, which are used widely for assessing the genomic actions of statins.[30–33]

The induction of simvastatin-induced HO-1 occurs through several known pathways. Unlike the other pleiotropic effects of statins, HO-1 induction does not appear to depend on the decreased formation of isoprenoids, which are important for small G-protein activation.[34] In addition, the HMG-CoA product, mevalonate, did not attenuate the HO-1 response to simvastatin, showing that the involvement of isoprenoid-dependent pathways, such as the blockade of Rho and its downstream target Rho kinase, are not involved (Fig. 4A).[35,36] This is further supported by the finding that an inhibitor of NOS (L-NAME) also had no effect on simvastatin-induced HO-1 expression (Fig. 4B). L-NAME should have reversed this effect if NO, which is one of the established HO-1 inducing signaling molecules,[37–39] was responsible for statin-dependent HO-1 induction. The addition of the L-NAME, however, did not attenuate the HO-1 response to simvastatin.

In general, the activation of MAPKs plays a key role in the induction of HO-1 gene expression.[40–44] The effects of pharmacological inhibitors of signaling intermediates on simvastatin-dependent HO-1 regulation were measured to identify the signal transduction pathways. The inhibition of HO-1 induction by specific inhibitors suggested that the PI3K/Akt pathway is involved in simvastatin-dependent HO-1 protein expression (Fig. 6).[19,28,40,42,45]

HO-1 mRNA and protein induction by simvastatin was associated with the protection of RPE cells from oxidative stress. ROS formation was reduced in RPE cells after a pretreatment with 10 μM simvastatin. The simvastatin-induced blockade of free radical formation was reversed in the presence of the HO inhibitor, ZnPP. And increased ROS formation was recovered in H_2O_2-treated human RPE cells after being treated with simvastatin. This effect was abolished by ZnPP, suggesting that HO-1 and its enzymatic products have functional relevance and are responsible for the observed antioxidative actions (Fig. 7).[19,46,47] Qian et al. recently demonstrated that statins protect ARPE cells from oxidative stress via an NADPH oxidase and/or p38 MAPK-dependent mechanisms.[29] Their study, however, missed the most important intermediate, HO-1. In addition, they did not evaluate other pathways, such as PI3K/Akt.

The pathophysiology of AMD is diverse, but chronic exposure to oxidative stress and aging are well-known risk factors. The prevention of AMD might be possible if the level of oxidative stress within the eye can be reduced. This study demonstrates that the antioxidant defense protein HO-1 is an intracellular site of action for simvastatin. In addition, the simvastatin-dependent upregulation of HO-1 occurs mainly via PI3K/Akt-dependent signaling pathways. This is the first study to reveal the simvastatin-induced modulation of oxidative stress defense enzyme, HO-1, in RPE cells in vitro. Simvastatin may have some clinical benefits in preventing the retinal diseases associated with oxidative stress, such as AMD.

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