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Impact of HMG-CoA Reductase Inhibition on Oxidant-Induced Injury in Human Retinal Pigment Epithelium Cells

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ABSTRACT

In addition to cholesterol-lowering effect, HMG-CoA reductase inhibition by statins has been shown to have protective effect in many cells type. The loss of vision in retinal degeneration disease associates with oxidative stress and apoptosis in retinal pigment epithelium (RPE) cell. This study was designed to examine the effect of statins on oxidant-induced damage in human RPE cells. Cultured human ARPE-19 (ARPE) cells were challenged with hydrogen peroxide (H_2O_2) plus tumor necrosis factor alpha (TNFα) in the presence or absence of statins or various stress signaling inhibitors, including anti-oxidants *N*-acetyl cysteine (NAC), the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenylene iodonium (DPI), and the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580. Apoptosis was evaluated by TUNEL analysis and cell viability was determined by MTT assay. Reactive oxygen species (ROS) were detected by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA). Expression of p-p38 MAPK protein was measured by Western blot analysis. Our findings indicate that statins treatment significantly suppressed oxidant-induced ROS accumulation and RPE apoptosis. Statins increased cell viability in a dose-dependent manner. In addition, statins treatment prevented the activation of NADPH oxidase and p38 MAPK signaling induced by oxidative stress. These results suggest that statins protects ARPE cells from oxidative stress via an NADPH oxidase and/or p38 MAPK-dependent mechanisms, which may contribute to statins-induced beneficial effects on RPE function. J. Cell. Biochem. 112: 2480–2489, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: APOPTOSIS; RPE; OXIDATIVE STRESS; ROS; p38

Retinal degenerative disease such as age-related macular degeneration (AMD) is the leading cause of blindness among the elderly population [Javitt et al., 2003; Augood et al., 2006]. Oxidative injury is thought to play a crucial role in dysfunction, or apoptosis of retinal pigment epithelium (RPE) cells in AMD [Winkler et al., 1999; Cai et al., 2000]. RPE cells are basically prone to oxidative stress and capable to produce reactive oxygen species (ROS) under certain conditions [Dorey et al., 1989; Miceli et al., 1994]. ROS induce oxidative damage in AMD, cataracts, and uveitis [Rao, 1990; Winkler et al., 1999; Truscott, 2000], and may promote potentially inflammatory processes [Kopprasch et al., 2003]. In accordance with the reduction of anti-oxidative production in aged RPE cells, oxidative stress is thought to contribute to the pathogenesis of AMD [Samiec et al., 1998].

Atorvastatin, an HMG-CoA reductase inhibitor, has been largely used to reduce morbidity and mortality associated with cardiovascular disease [Sever et al., 2004; Ye et al., 2006]. Statins has been shown to reduce RPE cellular cholesterol and inhibit secretion of apoB100, which are the backbone lipoprotein particles accumulating in drusen, the pathognomonic histopathologic Bruch's membrane lesions of AMD [Wu et al., 2010]. However, the beneficial effects of statins may extend beyond the cholesterol lowering mechanism. Statins have been reported to prevent oxidant-induced ROS production and apoptosis in endothelial progenitor cells [Urbich et al., 2005; Bao et al., 2010a] and the sciatic nerve [Gurpinar et al., 2010]. It has been suggested that atorvastatin (ATV) exerts anti-oxidant effects by downregulation of mRNA expression of essential nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit nox1 [Wassmann et al., 2002]. The NADPH oxidase system is the main source of ROS production [Miceli et al., 1994]. The increased ROS lead to increased oxidant stress and the activation of multiple signaling pathways, including p38 transduction signaling pathway to coordinate cellular responses [Martindale and Holbrook, 2002; Ho et al., 2006], all of which promote RPE cells

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dysfunction. The p38 signaling, a mitogen-activated protein (MAP) kinase, plays important role in regulating many cellular processes including inflammation, differentiation, apoptosis, and proliferation [Ono and Han, 2000]. Statin has been suggested to prevent p38 activation and protects the cells from oxidative injury [Bao et al., 2010b]. However, its role in retinal health has not been documented. Therefore, in the present study, we test the hypothesis that ATV protects RPE cell from injury initiated by oxidative stress, the underlying protective effects of ATV involve reducing oxidative stress, ROS production, and modulating p38 mitogen-activated protein kinase (p38 MAPK) activation in RPE cells.

MATERIALS AND METHODS

MATERIALS

Diphenylene iodonium (DPI), SB203580, NG-mono-methyl-L-arginin (LNMA), H_2O_2, and anti-β-actin were obtained from Sigma–Aldrich (St. Louis, MO). *N*-Acetyl cysteine (NAC) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) were purchased from Calbiochem (San Diego, CA). Tumor necrosis factor alpha (TNFα) was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against p38 were purchased from Upstate Biotechnology (Lake Placid, NY). MTT cell respiration assay kit was purchased from R&D system (Minneapolis, MN). Other reagents were indicated in the text.

HUMAN RPE CELL CULTURES

Human ARPE-19 cells (ARPE, American Type Culture Collection, Manassas, VA) were cultured and maintained at 37°C in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2. Cells between passages 3 and 11 were used for experiments. Cells were seeded at 1.2×10^4 cells/well in 200 μl medium for 96-well plate, 3.6×10^4 cells/well in 300 μl medium for chambered coverglass (Lab-Tek 136439; Miles, Napierville, IL), or 3×10^6 cells/dish in 10 ml medium for 100 mm plate. ARPE cells were grown in plates until 90% confluent.

CELL VIABILITY ASSAYS

Upon reaching the desired confluence, growth medium was removed and replaced with MEM supplemented with 0.5% FBS containing various reagents. The statins such as ATV and Simvastatin (SIM, EMD, Gibbstown, NJ) were evaluated. Both ATV and SIM were solubilized in DMSO and added to medium at different concentrations. To trigger oxidative stress and apoptosis, ARPE cells were stimulated with one exposure of 200, 400, or 600 μM H_2O_2 and 10 ng/ml TNFα according to the experimental design. Cell viability was estimated using a tetrazolium bromide (MTT)-based colorimetric method. Briefly, 10 μl of a stock solution of MTT (0.5 μg/μl) was added to each plate. After 4 h of incubation with MTT (at 37°C in a humidified atmosphere, 5% CO_2), 100 μl of HCl 0.01 M in 10% SDS was applied to solubilize the purple formazan for overnight. The OD value was measured with an ELISA reader at 550 nm. In viable cells, the mitochondrial enzyme succinate dehydrogenase can metabolize MTT into a formazan dye that absorbs light at 550 nm.

APOPTOTIC CELLS DETECTION BY IN SITU END-LABELING AND NUCLEAR STAINING

ARPE cells were seeded in chambered slides and treated with various reagents, respectively. Apoptotic cells were determined using TUNEL Apoptosis Detection Kit (Roche, Indianapolis, IN) described before [Ye et al., 2010a]. The apoptotic nuclei were labeled using terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer's instructions. The nuclei of apoptotic and non-apoptotic cells were counterstained with DAPI (0.1 μg/ml). The labeled cells were counted under a fluorescence microscope. The percentage of apoptotic cells was calculated as the ratio of TUNEL-positive cells to the DAPI-stained total cells, counted in six different random fields.

CASPASE-3 ACTIVITY MEASUREMENT

Caspase-3 activity was measured with an assay kit (Caspase-3/CPP32 Fluorometric Protease; Bio Vision, Mountain View, CA). ARPE cells were pre-incubated with different concentrations of ATV (0, 0.1, 1, 2.5, and 5 μM) or SIM (0, 1, 5, and 10 μM) for 2 h before H_2O_2/TNFα stimulation for set periods ranging from 0 to 16 h. Caspase-3 activity was performed using a colorimetric activity assay kit according to the manufacturer's instructions. The cells were harvested and then suspended in the cell lysis buffer to obtain cell lysate. Protein concentration was determined using Lowry Protein Assay and 200 μg protein of cell lysate was incubated in 100 μl of reaction buffer containing 5 μl of caspase-3 substrate (4 mM DEVD-pNA) in 96-well plates. The reaction buffer contained 1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NAC and 10% glycerol. The samples were incubated in the dark and caspase-3 activity was evaluated using a spectrophotometer at 405 nm.

MEASUREMENT OF ROS PRODUCTION

To evaluate ROS produced by RPE, ARPE cells were pre-incubated with different concentrations of statins or various inhibitors for 2 h before H_2O_2/TNFα stimulation for different time period, and then labeled with 20 μM of H_2DCFH-DA in serum-free medium at 37°C for 1 h. H_2DCFH-DA is converted to DCFH in the cell, and in the presence of ROS, it is further converted to the fluorescent product, dichlorofluorescein (DCF). DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Nonspecific fluorescence values without cells were subtracted from the fluorescence values with cells. ROS was determined by comparing the changes in fluorescence intensity with the control.

DETERMINATION OF NADPH OXIDASE ACTIVITY

The lucigenin-derived enhanced chemiluminescence assay was used to determine NADPH oxidase activity in ARPE cells. Cells were serum starved for 24 h and treated as indicated, washed twice with ice-cold PBS and harvested. After low spin centrifugation, the pellet was resuspended in buffer containing 1 mM ethylene glycol tetraacetic acid (EGTA), protease inhibitors, and 150 mM sucrose. Then, the cells were lysed. Two hundred micrograms of protein sample including 5 μM lucigenin was measured over 6 min in quadruplicate using NADPH (100 μM) as a substrate in a

luminometer (Berthold luminometer centro LB 960, Germany). Data were collected at 2 min intervals in order to measure relative changes in NADPH oxidase activity.

WESTERN BLOT ANALYSIS

Following treatment, cells were washed with ice-cold PBS and lysed as described previously [Ye et al., 2010b]. Samples were homogenized in lysis buffer (in mmol): 25 Tris–HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na_3VO_4, 1% Triton X-100, 2% SDS, and 1% protease inhibitor cocktail. The protein concentration was determined by the Bradford method. Fifty micrograms of protein was loaded in each lane and subjected to 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were probed with antibodies against phospho-p38 MAPK. Horseradish peroxidase-conjugated secondary antibodies were used in conjunction with an ECL *chemiluminescence* detection system (NEN Life Science Products, Boston, MA). Protein signals were quantified by densitometry analysis using Image J software and normalized to the corresponding β-actin signal.

STATISTICAL ANALYSIS

The obtained data were presented as means ± standard deviations for replicate experiment. One-way ANOVA in GraphPad Prism 5.0 software were used for statistical analysis and $P < 0.05$ was considered as statistically significant.

RESULTS

STATINS PREVENTED H_2O_2-INDUCED CELL DEATH AND APOPTOSIS OF ARPE CELLS

To determine the cytotoxicity of statins on human RPE cells, MTT assay was carried out with various doses of statins. As shown in Figure 1A, up to 5 μM for ATV or 10 μM for SIM, statins did not affect the cell viability of ARPE cells. To observe the effects of statins on RPE cells viability in response to oxidative damage, ATV or SIM was added 2 h before H_2O_2/TNFα stimulation for 16 h. No change was showed in cells viability with 200 μM H_2O_2/TNFα group compared to control (Fig. 1B). Previous studies indicated that 200 μM H_2O_2/TNFα does not affect ARPE cells viability [Tsao et al., 2006; Faghiri and Bazan, 2010]. It has been suggested that the persistent elevation of phosporylated-Akt induced by 200 μM H_2O_2, may enhance the ARPE cell's ability to resist the damaging effects of low level oxidative stress [Faghiri and Bazan, 2010]. However, cell viability of ARPE was significantly decreased by 400 μM H_2O_2 plus 10 ng/ml TNFα. As demonstrated in Figure 1B, cell viability was increased by ATV or SIM in a dose-dependent manner, and the maximal inhibitory effect was reached at 5 μM for ATV and 10 μM for SIM, suggesting that statins effectively prevented ARPE cells from H_2O_2-induced cell death. The effects of statins on survival of ARPE cells were partially reversed by the product of the HMG-CoA reductase, mevalonate (Fig. 1C). Consistently, statins inhibited ARPE cell apoptosis induced by H_2O_2 plus TNFα in a dose-dependent manner and the maximal inhibitory effect was reached at 5 μM for ATV and 10 μM for SIM (Fig. 1D). The anti-apoptosis effects of ATV and SIM were partially reversed by mevalonate (Fig. 1E). ATV, SIM, or mevalonate alone has no effect.

STATINS SUPPRESSION H_2O_2/TNFα-INDUCED CASPASE-3 ACTIVITY

To determine the mechanism by which statins protects human RPE cells from oxidative stress, the inhibitory effect of statins on caspase-3 activity was performed. Caspase-3 activity increased to 4.4-fold compare to control at 9 h and was sustained to 16 h after treated with 400 μM H_2O_2 plus 10 ng/ml TNFα (Fig. 2A). The increased activity of caspase-3 was significantly inhibited by 5 μM ATV or 10 μM SIM. In addition, DAPI staining showed strong fluorescent spots (apoptotic nuclear) in H_2O_2/TNFα-treated ARPE cells. The DNA fragmentation was almost abrogated completely by 5 μM ATV or 10 μM SIM (Fig. 2B).

ATV INHIBITED ROS PRODUCTION INDUCED BY H_2O_2

The intracellular ROS levels were measured by the ROS-sensitive fluorescent probe, H_2DCFH-DA using a flow cytometry. Dose–response data for intracellular ROS accumulation by H_2O_2 (0, 200, 400, 600, or 800 μM) plus 10 ng/ml TNFα are shown in Figure 3A. H_2O_2 increased DCF fluorescence was effectively reduced by 5 μM ATV or 10 μM SIM treatment (Fig. 3B). To determine whether the inhibitory roles of statins in H_2O_2-induced RPE cells apoptosis involved ROS, we observed the effects of the free radical scavenger (NAC, 1 mM), the NADPH oxidase inhibitor DPI (10 μM) and eNOS inhibitor (LNMA 1 mM) on RPE viability (Fig. 3C), apoptosis (Fig. 3D), and ROS generation (Fig. 3E). NAC or DPI pretreatment significantly increased RPE cells viability and reduced oxidant-induced cells apoptosis. Inhibition of the NOS by LNMA did not affect RPE apoptosis, suggesting a NADPH dependent, but not NOS-dependent, apoptotic signaling pathway, which is upregulated in this procedure. In addition, NAC or DPI pretreatment markedly prevented the ROS induced by H_2O_2. LNMA had no inhibitory effects, indicating ROS production upregulated via NADPH but not the NOS.

ATORVASTATIN INHIBITED H_2O_2-INDUCED ACTIVATION OF NADPH OXIDASE

To investigate the underlying mechanism by which statin suppressed intracellular oxidative stress, NADPH oxidase activity was measured. Challenge with 400 μM H_2O_2 TNFα plus 10 ng/ml led to a time dependent increase of NADPH oxidase activity to 227 ± 47% at 40 min and 301 ± 60% at 60 min (Fig. 4A). As demonstrated in Figure 4B, pretreatment of the cells with 5 μM ATV or 10 μM SIM reduced H_2O_2-dependent NADPH oxidase activation.

ATV INHIBITED RPE CELLS APOPTOSIS INDUCED BY H_2O_2/TNFα INVOLVING MAPK SIGNALING

Activation of p38 plays an essential role in H_2O_2-mediated cell death. To investigate whether p38 MAPK signaling was involved in protective effects of ATV on RPE cells, we observed the role of the p38 MAPK inhibitor, SB203580 (SB) in H_2O_2-induced cell death. ARPE cells were seeded in growth medium for 24 h. The culture medium was then replaced by 0.5% MEM medium. Cells were treated with or without 20 μM SB for 2 h before addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 16 h. Cell viability was then determined by

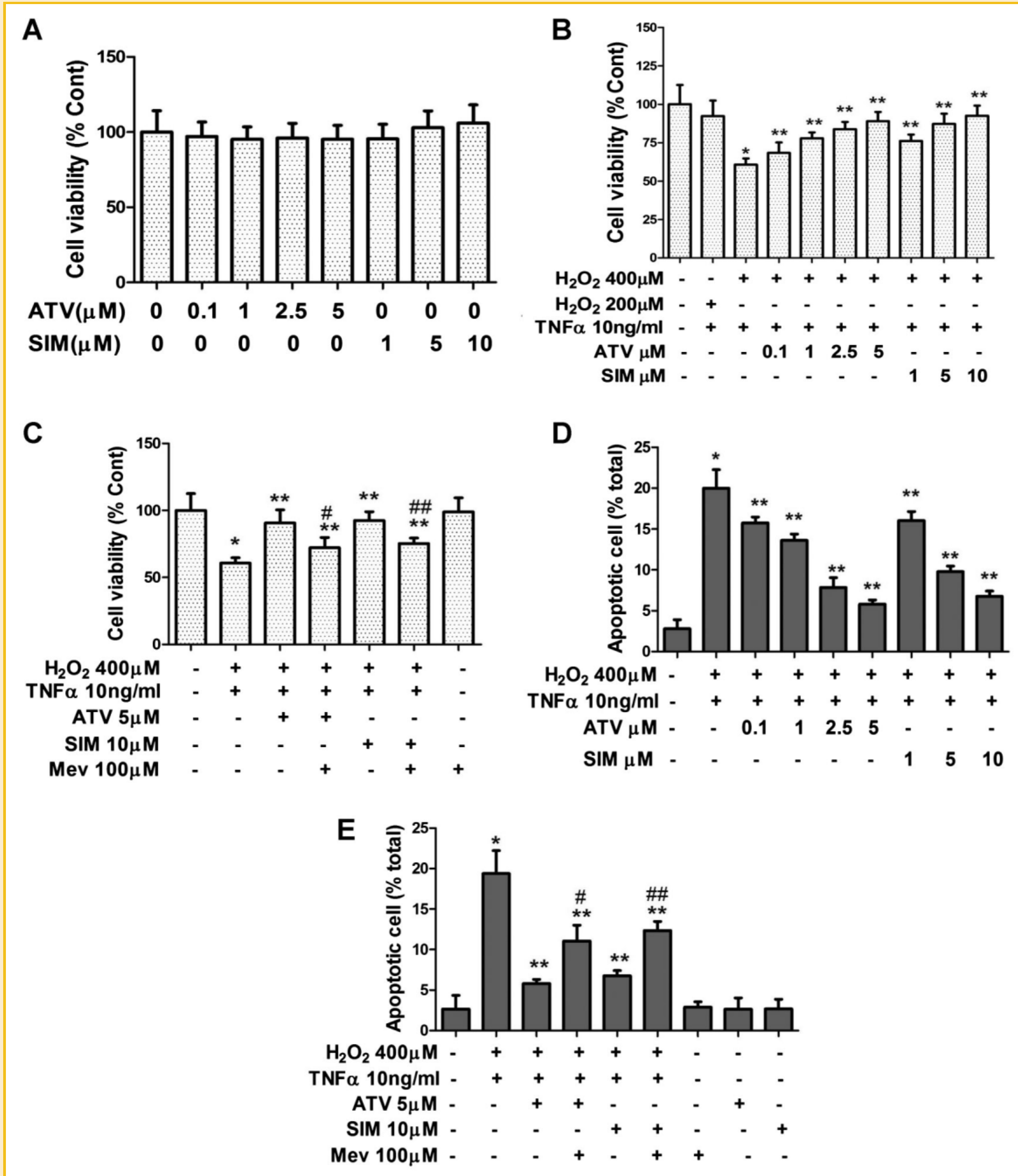

Fig. 1. Satins prevented cell death and apoptosis of human RPE cells induced by H_2O_2/TNFα. A: ARPE was incubated with various concentrations of ATV (0, 0.1, 1, 2.5, and 5 μM) or SIM (0, 1, 5, and 10 μM) for 16 h. Cell viability was detected by MTT. B: ARPE was pre-incubated with different concentrations of ATV or SIM for 2 h before treated with H_2O_2 (200 and 400 μM) plus 10 ng/ml TNFα for 16 h. Cell viability was detected by MTT. C: ARPE was pre-incubated with ATV (5 μM) or SIM (10 μM) with or without mevalonate (100 μM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 16 h. Cell viability was determined by MTT assay. D: ARPE was pre-incubated with different concentrations of ATV or SIM for 2 h before treated with 400 μM H_2O_2 plus 10 ng/ml TNFα for 16 h. Apoptosis was detected by TUNEL. E: ARPE was pre-incubated with ATV (5 μM) or SIM (10 μM) with or without mevalonate (100 μM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 16 h. Cell apoptosis was determined by TUNEL. Data are mean ± SD (n = 6). $^{*}P < 0.05$ versus untreated cells (Cont), $^{**}P < 0.05$ versus H_2O_2 /TNFα, $^{\#}P < 0.05$ versus ATV + H_2O_2/TNFα, $^{\#\#}P < 0.05$ versus SIM + H_2O_2/TNFα. ATV, atorvastatin; SIM, simvastatin; Mev, mevalonate.

MTT assay (Fig. 5A), and confirmed by the trypan blue exclusion and TUNEL assay (Fig. 5B,C). We found that SB partially blocked the effects of H_2O_2, which suggested that p38 MAPK was involved in RPE cells apoptosis. 20 μM SB alone had no effect, suggesting SB was not cytotoxicity to ARPE cells. Western blot was used to determine whether p38 phosphorylation can be induced by H_2O_2 plus TNFα exposure. Cells were treated with ATV (5 μM), SIM (10 μM), SB (20 μM), NADPH oxidase inhibitor DPI (10 μM), or the free radical scavenger (NAC, 1 mM) for 2 h before addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 2 h. Whole cell extracts were prepared and analyzed using antibodies against the phosphorylated p38. Phosphorylation of p38 was upregulated following treatment

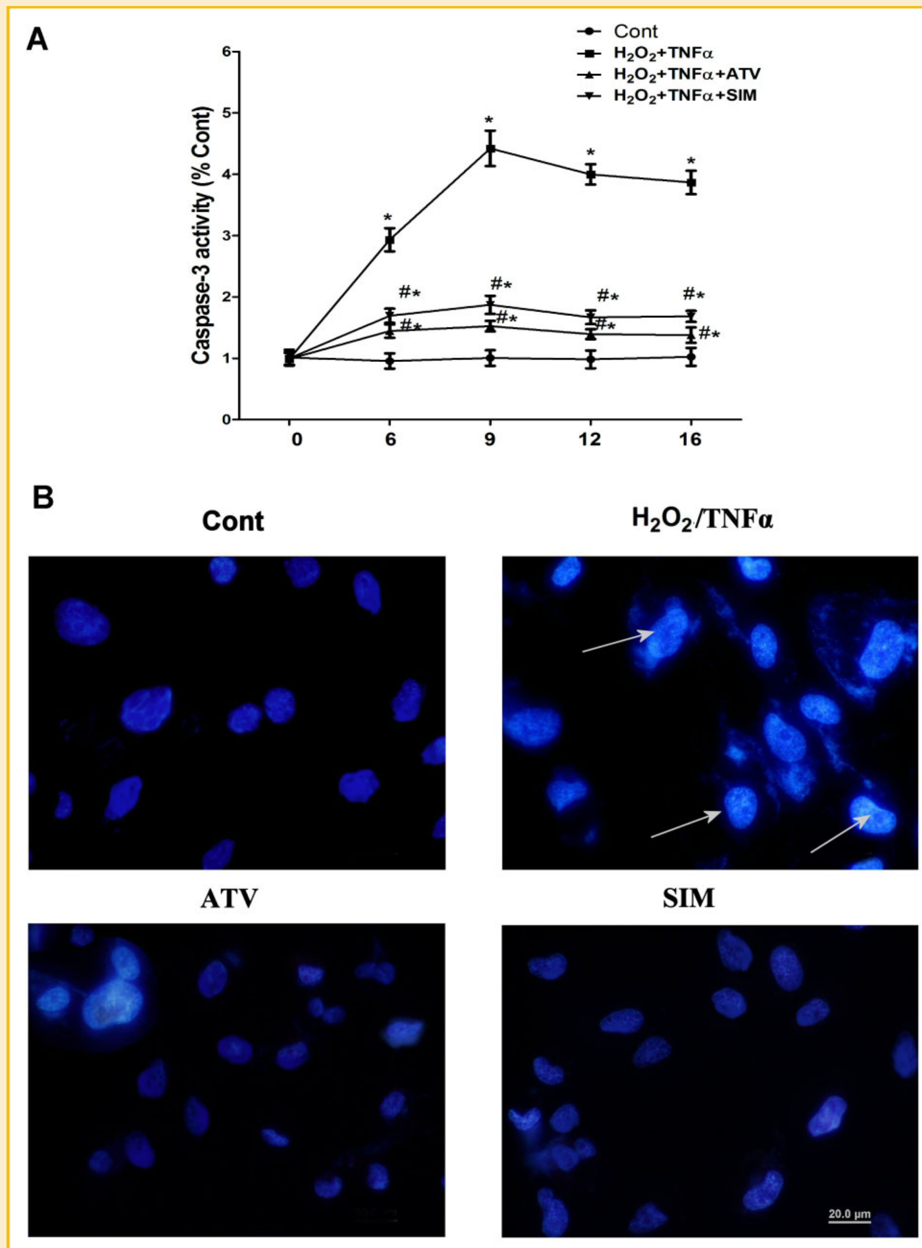

Fig. 2. Statins inhibited the caspase-3 activity induced by H_2O_2/TNFα. ARPE cells were treated with 400 μM H_2O_2 plus 10 ng/ml TNFα or 5 μM ATV or 10 μM SIM. A: Caspase-3 activation was examined during 16 h culture using caspase-3 activity assay kit. Six independent experiments were performed. $^{*}P < 0.05$ versus Cont, $^{\#}P < 0.001$ versus H_2O_2/TNFα. B: Apoptotic cells were visualized by using DAPI staining after 16 h exposure to 400 μM H_2O_2 plus 10 ng/ml TNFα. Strong fluorescent spots show apoptotic DNA cleavage. Representative pictures were selected from four independent experiments. Scale bar, 20 μm.

with 400 μM H_2O_2 plus 10 ng/ml TNFα (Fig. 5D). The activation of p38 MAPK induced by H_2O_2 was significantly suppressed by 5 μM ATV, 10 μM SIM, 10 μM DPI, 1 mM NAC, or 20 μM SB.

DISCUSSION

ATV REDUCES RPE CELLS DEATH AND APOPTOSIS

Statins have been shown to reduce cells death and improve cells function by lowering serum LDL via HMG-CoA reductase inhibition, eNOS upregulation and reduction of O_{2-} formation [Dobrucki et al., 2001]. The present study examines the anti-oxidant and anti-apoptosis effects of statins in human ARPE cells, both ATV and SIM significantly reduced RPE cell death and apoptosis without any cytotoxicity. The anti-apoptosis effect by statins was partially inhibited by the product of the HMG-CoA reductase, mevalonate. These data suggest that the protective effect of statins against oxidative stress involves combination effects other than inhibition of HMG-CoA reductase. The reduction in apoptosis by statins in RPE cells coincided with reduced activation of P38 kinase, suggesting that statins inhibit cell death related to inhibition of the P38 kinase.

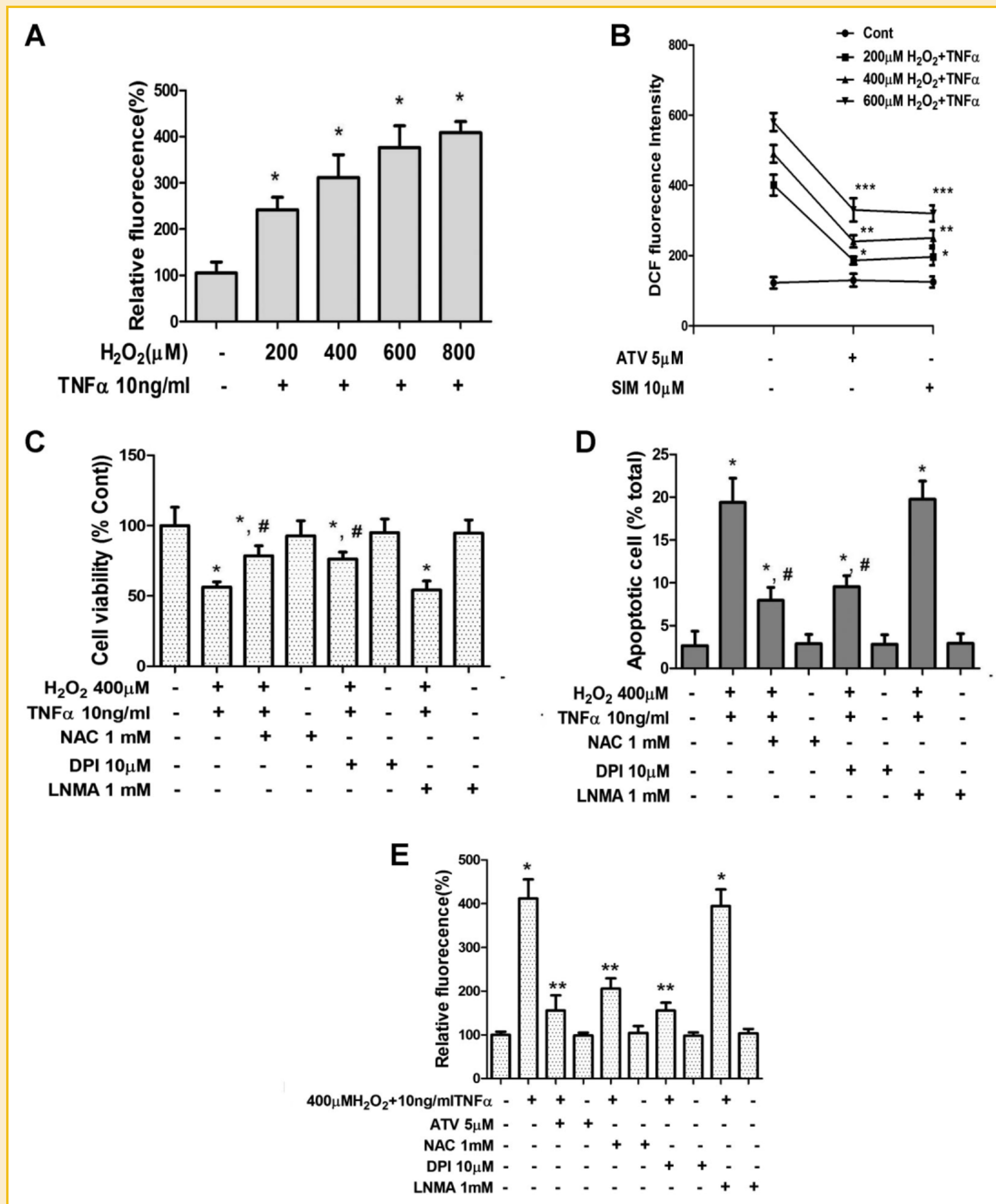

Fig. 3. ATV prevented ROS production. A: Cells were treated with H_2O_2 (200, 400, 600, or 800 μM) plus 10 ng/ml TNFα for 2 h and then labeled with 20 μM H2DCF-DA for 1 h at 37°C. ROS was measured by H2DCF-DA fluorescence. $^{*}P < 0.01$ versus untreated cells (Cont). Relative fluorescence of samples versus control is shown (all experiments n = 6, mean ± SD). B: Cells were treated with 5 μM ATV or 10 μM SIM for 2 h before addition of H_2O_2 (200, 400, or 600 μM) plus 10 ng/ml TNFα for 2 h and then ROS was determined. $^{*}P < 0.05$ versus 200 μM H_2O_2/10 ng/ml TNFα, $^{**}P < 0.05$ versus 400 μM H_2O_2/10 ng/ml TNFα, $^{***}P < 0.05$ versus 600 μM H_2O_2/10 ng/ml TNFα. C: Cells were pre-incubated with DPI (10 μM), NAC (1 mM) or LNMA (1 mM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 16 h and cells viability was detected. $^{*}P < 0.01$ versus Cont. $^{\#}P < 0.05$ versus H_2O_2/TNFα (experiments n = 8, mean ± SD). D: Cells were pre-incubated with NAC (1 mM), DPI (10 μM), or LNMA (1 mM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml for 16 h and apoptosis was detected. $^{*}P < 0.05$ versus Cont. $^{\#}P < 0.05$ versus H_2O_2/TNFα (experiments n = 6, mean ± SD). E: Cells were pre-incubated with ATV (5 μM), NAC (1 mM), DPI (10 μM), or LNMA (1 mM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml and ROS was determined (experiments n = 6, mean ± SD). $^{*}P < 0.01$ versus untreated group. $^{**}P < 0.01$ versus H_2O_2/TNFα.

Mitogen-activated protein kinases, including c-Jun NH2-terminal kinase (JNK), p38 kinase and extracellular signal-regulated kinase (ERK), have been found to respond to a variety of environmental stress and extracellular stimulants [Xia et al., 1995; Chang and Karin, 2001]. JNK and p38 activation play an important role in cell death signaling, whereas activation of ERK is essential for cell survival [Davis, 2000; Ono and Han, 2000]. The p38 MAPK is a downstream target of proinflammatory cytokines and oxidative stress [Hu et al., 1999]. In addition, high-level and persistent activation of JNK1 and p38 has been indicated to induce RPE cell

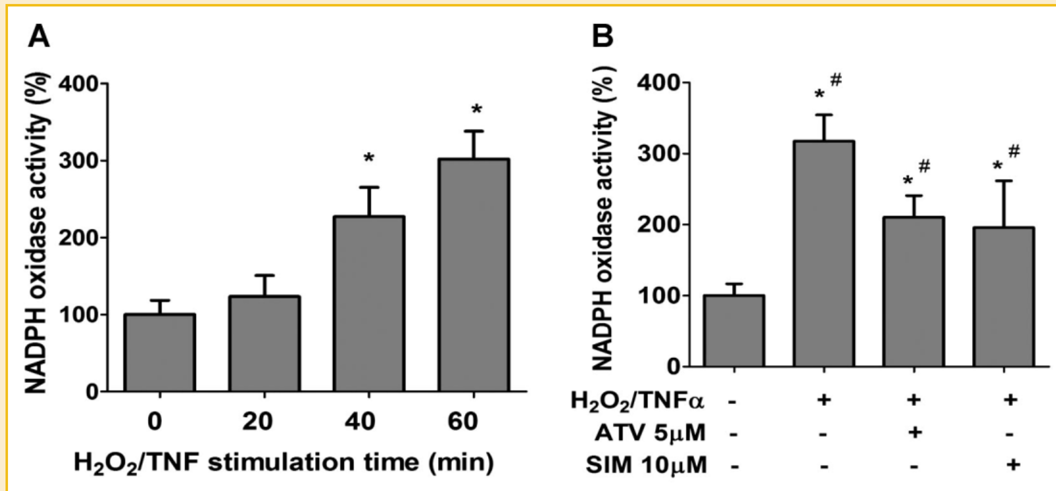

Fig. 4. Statins suppressed H_2O_2-induced activation of NADPH oxidase. A: ARPE was stimulated with 400 μM H_2O_2 plus 10 ng/ml TNFα, NADPH oxidase activity was measured at different time points (0, 20, 40, and 60 min) with lucigenin-enhanced chemiluminescence. B: Pretreatment of cells with 5 μM ATV or 10 μM SIM 2 h prior to 400 μM H_2O_2 plus 10 ng/ml TNFα, NADPH oxidase activation was assessed after 60 min using NADPH as a substrate. Results represent the percentage increase of chemiluminescence over buffer stimulation, and were expressed as means ± SD of four independent experiments, each performed in quadruplicate. $^*P < 0.01$ versus untreated cells. $^\#P < 0.05$ versus H_2O_2/TNFα.

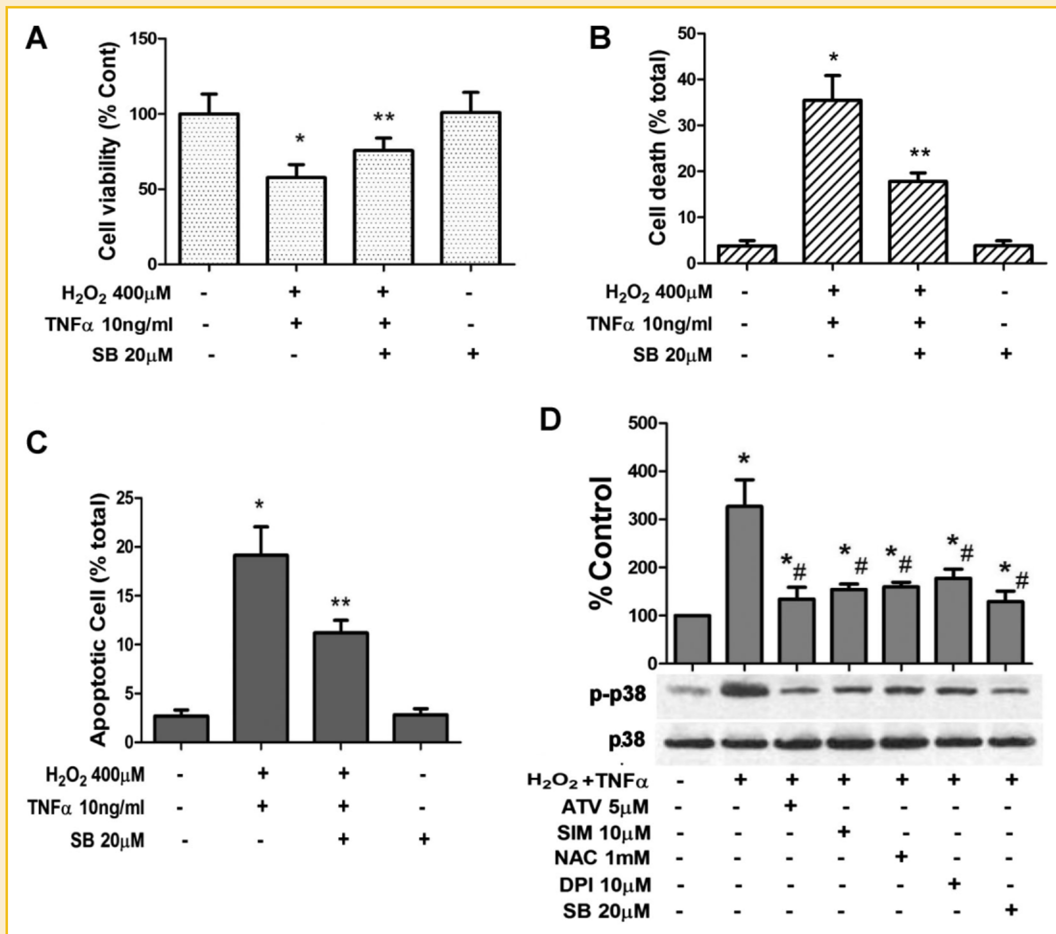

Fig. 5. Atorvastatin inhibited RPE apoptosis induced by H_2O_2 involving a MAPK mechanism. Cells were pre-incubated with the p38 inhibitor, SB203580 (20 μM) for 2 h before the addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 24 h and cells viability (A), trypan blue (B), and apoptosis (C) were determined. Data are expressed as means ± SD (n = 6). $^*P < 0.05$ control, $^{**}P < 0.01$ versus H_2O_2 plus TNFα group. D: Cells were pre-incubated with ATV (5 μM), SIM (10 μM), SB (20 μM), DPI (10 μM), or NAC (1 mM) for 2 h before addition of 400 μM H_2O_2 plus 10 ng/ml TNFα for 2 h and Western blot was performed. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Data are expressed as means ± SD (n = 4). $^*P < 0.05$ control, $^\#P < 0.01$ versus H_2O_2/TNFα group.

death during serum depletion and oxidative stress [Zhang and Jope, 1999; Hecquet et al., 2003; Roduit and Schorderet, 2008]. Taken together, these data suggest that the anti-apoptosis effects of statins involve inhibition of P38MAP activity. On the other hand, in RPE cells treated with H_2O_2, different apoptosis characteristics such as cytochrome *c* release, caspase-9, and/or caspase-3 activation have been detected [Yamakawa et al., 2000; Kim et al., 2003; Burkitt et al., 2004]. Our study also found that H_2O_2 plus TNFα increased the activity of caspase-3, which inhibited by ATV and SIM. Interestingly, statins taken up by the RPE cells to protect from oxidative stress-induced apoptosis showed increased cell viability, but no cytotoxicity of human RPE cells. Therefore, statins may be safely applied to the in vivo model without toxicity.

ATORVASTATIN INHIBITS INTRACELLULAR OXIDATION INDUCED BY H_2O_2

Evidence supports a strong association between oxidative stress and development of AMD [Cano et al., 2010; Hollyfield, 2010; Jin et al., 2010]. Transient fluctuations of ROS could serve some regulatory functions, whereas high and persistent levels of ROS damage mitochondrial DNA and lead to cells death [Cai et al., 1999]. The cytotoxicity of H_2O_2 on RPE is closely related with ROS accumulation, which is thought to play a key role in H_2O_2-induced RPE apoptosis [Miceli et al., 1994; Giddabasappa et al., 2010]. RPE cells are sensitive to ROS induction as compared with other cells type. Our study also showed that ROS are strongly involved in H_2O_2-induced RPE cells apoptosis, since radical scavengers NAC reduced apoptotic cell death. Statins has pleiotropic effects including anti-atherogenic and anti-inflammatory actions [Lefer et al., 2001]. Beyond its cholesterol lowing effects, statins has been found to inhibit the in vitro oxidation of LDL and to prevent various cells from oxidative stress injury [Rubba, 2007]. In this study, statins significantly rescued human RPE cells from H_2O_2-induced cell death. H_2O_2 strongly enhanced intracellular ROS production in human RPE cells that was significantly inhibited by statins treatment. Thus, accumulating evidence indicate that ATV could serve as survival factor directly or indirectly associated with the anti-apoptotic pathway.

THE POTENTIAL PHARMACOLOGICAL ROLE OF STATINS IN THE RPE AND RETINAL DISEASE

Oxidative stress contributes to the pathogenesis of retinal degeneration, optic nerve pathologic conditions, and inflammatory in optic neuritis [Winkler et al., 1999; Hollyfield et al., 2008; Williams, 2008]. The RPE is at high risk for oxidative stress because it resides in high oxygen and phototoxic blue light environment [Algvere et al., 2006]. In RPE cells, oxidative stress was significantly augmented after exposure to H_2O_2, *t*-butyl hydroxide, and ultraviolet light [Dontsov et al., 1999; Patton et al., 2002; Kubota et al., 2009; Giddabasappa et al., 2010]. Oxidative stress to RPE cell layer over time is likely to be a potential stimulus for inducing cellular inflammatory events and cell damage [Kubota et al., 2009]. Limiting oxidative damage to the RPE represents one strategy to reduce the risk for developing retinal disease. Beyond the cholesterol reduction, statins has been proved to reduce oxidative damage, increase cell viability, and decrease cell apoptosis [Bao et al., 2010b]. ATV inhibits choroidal neovascularization induced by laser photocoagulation in mice involving reducing macrophage infiltration into the RPE [Yamada et al., 2007]. ATV or lovastatin administrated following the clinical onset has therapeutic effect in experimental autoimmune uveoretinitis [Kohno et al., 2007]. To date, the physiological accumulation of statins in the RPE has not been investigated. In the present study, the anti-oxidant ATV significantly reduce oxidation and apoptosis of human RPE cells. The combination of these effects may explain the beneficial effects of statins on the progression of retinal disease. Statins was well tolerated by a broad range of patients, and its safety profile has been investigated in this extensive clinical program [Shepherd et al., 2004; Sang et al., 2009].

In summary, our findings strongly suggested that statins exerted its anti-apoptosis activity through inhibiting NADPH/ ROS/p38 signaling in human ARPE-19. Our observations indicate that statins can protect RPE from oxidative stress through multiple mechanisms. Based on this available evidence, statins may play a special role in responding to the local redox environment of human RPE cells and may be considered as a therapeutic approach to degenerative eye disease such AMD.

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