Possible Role of HMG CoA Reductase Inhibitor on the Oxidative Stress induced by Advanced Glycation Endproducts (AGEs) in Vascular Smooth Muscle Cell of Diabetic Vasculopathy

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Directed by Professor Hyuck Moon Kwon

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This certifies that the Doctoral Dissertation of Se-Jung Yoon is approved.

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I dedicate the dissertation to my parents and my loving family.

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ABSTRACT

Possible Role of HMG CoA Reductase Inhibitor on the Oxidative Stress induced by Advanced Glycation Endproducts (AGEs) in Vascular Smooth Muscle Cell (VSMC) of Diabetic Vasculopathy

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(Directed by Professor Hyuck Moon Kwon)

Antioxidative effect of HMG CoA reductase inhibitor has recently been noted as one of their pleiotrophic effects and is especially important in the aspects of diabetic vasculopathy. Advanced glycation endproducts (AGEs) induced smooth muscle cell proliferation and formation of reactive oxygen species (ROS) are emerging as one of the important mechanism of diabetic vasculopathy, but little is known about antioxidative action of HMG CoA reductase inhibitor on AGEs. We hypothesized that HMG CoA reductase inhibitor might reduce the AGEs-induced intracellular ROS of VSMCs. In this study, we analyze the possible mechanism of action of HMG CoA reductase inhibitor in the AGEs-induced cellular signaling. Rat aortic smooth muscle cell (RASMC) culture was done using the different levels of AGEs stimulation in the presence or absence of HMG CoA reductase inhibitor. Increasing

concentration of AGEs stimulation was associated with increased VSMC proliferation and with increased ROS formation. Increased NFκB, phosphorylated ERKs, phosphorylated p38, COX-2 and c-jun by AGEs stimulation were noted. Comparing with the control cells (without HMG CoA reductase inhibitor treatment), HMG CoA reductase inhibitor inhibited AGEs-stimulated VSMC proliferation in dose dependent manners. HMG CoA reductase inhibitor also inhibited intracellular ROS formation and the expression of NFκB, phosphorylated ERKs, phosphorylated p38, COX-2 and c-jun induced by AGEs. Neointimal formation after balloon injury was much thicker in streptozocin-indcued diabetic S-D rats than in sham-treated group, but less neointimal growth was observed in those treated with HMG CoA reductase inhibitor after balloon injury. siRNA-mediated silencing of RAGE (receptor for AGEs) expression had no effect on the change of ERK mediated by HMG CoA reductase inhibitor. In vitro and in vivo data suggest that AGEs may play a key role in VSMC proliferation and increase the oxidative stress. HMG CoA reductase inhibitor inhibited the AGEs-induced proliferation of VSMCs, ROS formation and activity of the MAPK. Activation of MAPK system and increased ROS formation may be the possible mechanism of diabetic vasculopathy induced by AGEs and HMG CoA reductase inhibitor may play a key role in treatment of AGEs-induced diabetic atherosclerosis.

Key words : AGEs, ROS, VSMC proliferation, HMG CoA reductase inhibitor, NFκB, ERK, p38, c-jun, COX-2

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I. INTRODUCTION

Factors underlying accelerated atherosclerosis in diabetes extend beyond dyslipidemia, hypertension and obesity. Even after correction of these typical risk factors such as hypercholesterolemia and rigorous control of glycemia, diabetic patients continue to experience increased atherosclerotic vascular disease[1-3]. Advanced glycation endproducts (AGEs), resulting from irreversible non-enzymatic glycation and oxidation of proteins or lipids, accumulate continuously on long-lived vessel wall proteins with aging and at an accelerated rate in diabetes. AGEs have been linked to the development of diabetic complications irrespective of other conventional risk factors[4]. Recent studies

have demonstrated that AGEs and its receptor/ligand interaction play a key role in neointimal formation after vascular injury irrespective of diabetes status[5-7]. AGEs stimulates the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB)-dependent pathways and subsequently induce the proinflammatory mediators and increased oxidative stress[8].

Reactive oxygen species (ROS), generated either extra- or intracellularly through ligand-receptor interactions, can function as signal transduction molecules and play a key role in the inflammatory process and atherosclerosis[9,21]. Various types of cells such as fibroblasts, endothelial cells, and vascular smooth muscle cells (VSMC) produce ROS in response to cellular activation signals[10]. Thus, ROS activate several transcription factors including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)[11]. This activation in turn leads to the coordinated expression of different genes that encode proteins such as cytokines, chemokines, adhesion molecules, and enzymes involved in the initiation and perpetuation of the inflammatory response[12].

HMG CoA reductase inhibitors, statins are effective lipid-lowering agents. Recent experimental studies suggested that the beneficial effects of HMG CoA reductase inhibitors in patients at risk for cardiovascular disease are not only a result of an improved lipid profile but also mediated by direct vasculoprotective actions[13,22]. Antioxidative effects of HMG CoA reductase inhibitors have recently been noted as one of their pleiotrophic effects. HMG CoA reductase inhibitors reduce intracellular ROS in endothelial cells by S-nitrosylation of thioredoxin[14]. Recently, several reports have suggested that HMG CoA reductase inhibitors could act as antioxidants in experimental atherosclerosis in rabbits[15], inhibiting the oxidation of LDL by activated monocyte-derived macrophages[16]. In rats, HMG CoA reductase inhibitors reduces ROS production[17] and prevents cardiac myocyte hypertrophy[18]. And there is also another recent article that shows the possible mechanism of antioxidative effect of HMG CoA reductase inhibitors. It revealed that HMG CoA reductase

inhibitors inhibit p38 MAPK and consequently inhibit the membrane translocation of the small G protein family members (Ras1- and RhoA-)[19]. However, little is known about the molecular mechanism of these drugs as antioxidants.

Antioxidant effect of HMG CoA reductase inhibitors is especially important in the aspects of diabetic complications such as macrovascular or microvascular vasculopathy. AGEs induced smooth muscle cell proliferation and oxidative stress formation is emerging as one of the important mechanism of diabetic vasculopathy, but little is known about action of pleiotrophic HMG CoA reductase inhibitors on AGEs. Therefore, in this study, we hypothesized that HMG CoA reductase inhibitors might reduce the AGEs induced intracellular ROS and inhibit p38 MAPK and consequently inhibit the membrane translocation of the small G protein family members in VSMCs and we also analyzed the possible mechanism of action of HMG CoA reductase inhibitors in the ROS-induced cellular signaling.

This study was performed to 1) evaluate the effects of HMG CoA reductase inhibitors on the proliferative cascade of vascular smooth muscle cells, 2) explore the effects of HMG CoA reductase inhibitors on ROS production in AGEs-stimulated proliferated VSMCs, 3) investigate the mechanism of dose-dependent modulation of HMG CoA reductase inhibitors on MAPK and NF-κB activity in VSMC proliferation induced by AGEs .

II. MATERIALS AND METHODS

1. Materials and experimental animals

A. Experimental materials

Amonng HMG CoA reductase inhibitors, Simvastatin was a generous gift from MSD (Merck & Co., Inc., New Jersey, USA, Ltd.). All other materials were purchased from commercial suppliers.

B. Experimental animals

6-8 weeks-old Spraque-Dawley rats (200g initial weight) were housed in a temperature-controlled environment under a 12-h light/dark cycle and were given standard chow and water. They were injected streptozocin (STZ, 80mg/kg) via intraperitoneal route and hyperglycemia was confirmed by using blood glucose meter (Accu-Chek Advantage, Boehringer Mannheim, Mannheim, Germany) within 1 week post-STZ and diabetic models were confirmed by blood glucose levels over 250 mg/dl. Rats treated with STZ that did not demonstrate significant elevation of blood glucose (<250 mg/dl) were excluded from the studies. Animals were handled in compliance with the Guiding Principles in the Care and Use of Animals. All rats were fed standard rat chow until they were 16 weeks of age. HMG CoA reductase inhibitor of 20 mg/kg, was mixed with pulverized standard chow, and 5 rats were treated for 8 weeks from 8^{th} week. After 8 weeks, carotid artery balloon injury was performed and after 4 weeks of more treatment, the rats were sacrificed for analysis.

2. Methods

A. Isolation of rat aortic smooth muscle cells (RASMC) and cell culture

RASMCs were isolated as previously described[13]. The thoracic aortas from 6- to 8-week-old rats were removed and transferred in serum-free Dulbecco's modified Eagle' medium (DMEM; Invitrogen Co, Carlsbad, CA, USA) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. The aorta was freed from connective tissue, transferred into Petri dish containing 5 ml of

an enzyme dissociation mixture containing DMEM with 1 mg/ml of collagenase type I (Sigma, St. Louis, MO, USA) and 0.5 μg/ml elastase (USB Bioscience, Cleveland, OH, USA), and incubated for 30 min and at 37°C. The aorta was the transferred into DMEM and the adventitia was stripped off with forceps under a binocular microscope. The aorta was transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 h at 37°C. The suspension was centrifuged (1,500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% fetal bovine serum (FBS). Rat aortic VSMCs were cultured in DMEM supplement with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin in 75-cm^2 flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (Forma Scientific, Inc., Marjetta, OH, USA.).

B. RASMC proliferation assay

Cellular proliferation was measured by PreMix WST-1 Cell Proliferation Assay System (TAKARA Biomedicals, Shiga, Japan). This system enables the measurement of cell proliferation with colorimetric assay, and bases on the cleavage of slightly red tetrazolium salt (WST-1) by mitochondrial succinate-tetrazolium reductase in viable cells. As the increase in enzymes activity leads to an increase of the production of formazan dye, the quantity of formazan dye is related directly to the number of metabolically active cells in the medium. Cells ($5\text{-}7\times10^3$) were seeded into wells of a 96-well culture plate and incubated with AGEs for the times indicated. WST-1 cell proliferation reagent was added directly to the supernatant (10 μl/100 μl growth medium), and incubated at 37℃ for 3h. The absorbancy of the solubilized dark red formazan product was then determined at 450nm.

C. AGEs and HMG CoA reductase inhibitors treatment

Cultured RASMC was co-incubated with prepared BSA-AGEs solution

(Sigma, St. Louis, MO, USA) by each concentration of 1, 10, 50, 100 and 1000 μg/ml for 2 hours at 37℃ for 3h. Simvastatin (supplied by Merck & Co., Inc., New Jersey, USA, Ltd.) was pretreated for inhibition experiment. AGEs stimulated RASMC was incubated with 0.1, 1, 5, 10, 20, 50 and 100 μM of activated HMG CoA reductase inhibitor for 1h before AGEs stimulation. HMG CoA reductase inhibitor was activated via alkaline hydrolysis as previously described[20].

D. Measurement of intracellular ROS generation

RASMCs were labeled with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probe, CA, USA) as previously described[14]. The probe H_2DCFDA (5M) entered the cell and the acetate group on H_2DCFDA was cleaved by cellular esterases, trapping the non-fluorescent 2′,7′ -dichlorofluorescin (DCF) inside.

Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled rat aortic smooth muscle cells were examined using a luminescence spectrophotometer for oxidized dye. The quiescent cells were treated with AGEs for 3 h before labeling with H_2DCFDA.

E. SDS–PAGE and Western blot analysis for NF-κB, p-ERK and p-P38.

Growth-arrested VSMCs were rinsed with cold phosphate-buffered saline (PBS) and immediately scraped into 1.5-ml tubes at −80 °C. Harvested cells were solubilized in a lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and centrifuged at 12,000 g for 20 min at 4°C. The protein concentrations of the supernatants were determined using a

Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates containing equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis, and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking in 10 mM Tris–HCl buffer (pH 8.0) containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, the membrane was treated with NF-κB, p-ERK and p-P38 primary antibodies (Santa Cruz Biotechnology, CA. USA), followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen–antibody bands were detected using an enhanced chemiluminescence reagent kit (Amersham Bioscience, Uppsala, Sweden) and quantified by densitometry.

F. RNA interference for RAGE mRNA

For function-blocking experiments, small interfering RNA molecules (siRNA) targeted at RAGE (receptor for AGEs) mRNA was used. A 21-nt sequence for siRNA was derived from the rat RAGE (GenBank accession no. GI: 81722) and obtained from Ambion, Inc. (TX, USA): small interfering RNA against RAGE (sense, 5'-GCUAGAAUGGAAACUGAACtt-3'; antisense, 5'-GUUCAGUUUCCAUUCUAGCtt-3'). Rat aortic smooth muscle cells were transfected with si-RAGE duplexes by using siPORT NeoFX (Ambion Inc., TX, USA).

Briefly, RNA duplex (10 nM of final concentration) was incubated in serum-free DMEM containing 15 μl of siPORT NeoFX for 10 min. The complex was added to the empty 60mm culture plate and then overlay SMC suspension (1 x 10^5 cells per plate) onto the culture plate wells containing transfection complexes and the transfected cells were incubated in normal cell culture conditions until ready for assay.

G. RT-PCR analysis for COX-2 and c-jun

The expression levels of various proteins were analyzed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was prepared by the Ultraspect™-II RNA system (Biotecx Laboratories, Inc., TX, USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 μg of total RNA, 1X reverse transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 μg of oligo(dT)15 and 15 units of AMV reverse transcriptase was incubated at 42℃ for 15 min, heated to 99℃ for 5 min, and then incubated at 0-5℃ for 5 min. PCRs were performed for 35 cycles with 3' and 5' primers based on the sequences of various genes. Primers were as follows: *c-jun*: 5'-AACGACCTTCTACGACGATG-3', 5'-GCAGCGTATTCTGGCTATGC-3' (364 bp); COX-2: 5'-TCCAATCGCTGTACAAGCAG-3', 5'-TCCCCAAAGATAGCATCTGC-3' (230 bp). GAPDH (primers 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' (450 bp)) was used as the internal standard. The signal intensity of the amplification product was normalized to its respective GAPDH signal intensity.

H. Induction of neointimal formation after balloon denudation in diabetic rat carotid artery

Balloon denudation of the common carotid artery endothelium was evoked in diabetic 16-weeks-old Spraque-Dawley rats (400g). Under isoflurane (Abbott Laboratories, St. Laurent, QC) anesthesia, a neck midline incision was made and, after exposure of the left carotid artery, a 2F Fogarty balloon catheter (Edwards Lifesciences, Mississauga, ON) was inserted into the external carotid branch to the aortic arch, insufflated (with about 0.2 ml saline), to produce slight resistance and withdrawn three times. After 4 weeks, they were compared

with sham-operated controls in which the same procedure was performed, except balloon insertion. Animal housing and experimentation in accordance with Animal Care and NIH guidelines were approved by the local animal care committee.

I. Perfusion of vessels, H&E staining and immunochemistry for COX-2 in rat carotid artery

The heart was removed from the anesthetized rat and quickly cannulated onto the Langendorff perfusion apparatus. The heart was perfused with Krebs-Ringer Bicarbonate Buffer (KRBB : 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 20 mM MOPS) for 10 min to wash out the blood and then fixed with 10% formalin.

The carotid artery was sliced transversely. Within 24 h after fixation, each sections were embedded in paraffin. Serial 2 μm artery sections were cut with microtome and mounted on siliconized slides. Paraffin sections were stained with hematoxylin and eosin (HE) and evaluated with light microscopy to assess the histological effects. Normal and neointimal areas were measured by using NIH images.

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen (LN2). Serial sections were prepared and immunohistochemistry to detect COX-2 was performed using (polyclonal monospecific antibodies to COX-2 (Abcam plc., Cambridge, UK).

J. Tissue preparation, H-E staining and immunochemistry for RAGE in diabetic human atheroma specimens.

Human carotid endarterectomy specimens were obtained from diabetic patients undergone carotid endarterectomy. Each specimen was fixed with 10% buffered formalin and embedded in paraffin. Immunohistochemistry was performed using polyclonal monospecific antibodies to RAGE (Santa Cruz Lab.

CA, USA). Peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) was used to visualize the sites of primary antibody binding to the antigen.

K. Statistical analysis

Data are presented as mean ± SEM. Differences between two experimental groups are examined by use of the Student's t test. One-way ANOVA followed by post hoc paired comparisons for comparison of multiple groups and ANOVA for repeated measures are used as appropriate (SPSS software). A value of $p<0.05$ is considered significant.

III. RESULTS

1. Effect of AGEs and HMG CoA reductase inhibitor on vascular smooth muscle cell proliferation

In order to investigate whether HMG CoA reductase inhibitor affects RASMC proliferation upon AGEs treatment, RASMCs were treated with AGEs in the presence or absence of HMG CoA reductase inhibitor and a cell proliferation assay was performed. As shown in Figure 1A, AGEs-induced increase in cell proliferation and the augmentation of cell proliferation induced by 50 μg/ml of AGEs were concentration-dependent (7.2 ± 2.4 fold increase compared to control at 50 μg/ml of AGEs). The maximal response was obtained 50 μg/ml of AGEs. HMG CoA reductase inhibitor inhibited the augmentation of cell proliferation induced by AGEs in dose-dependent manners (4.7 ± 1.2 fold reduction compared to AGEs-treated group at 10 μM of HMG CoA reductase inhibitor) (Figure 1B).

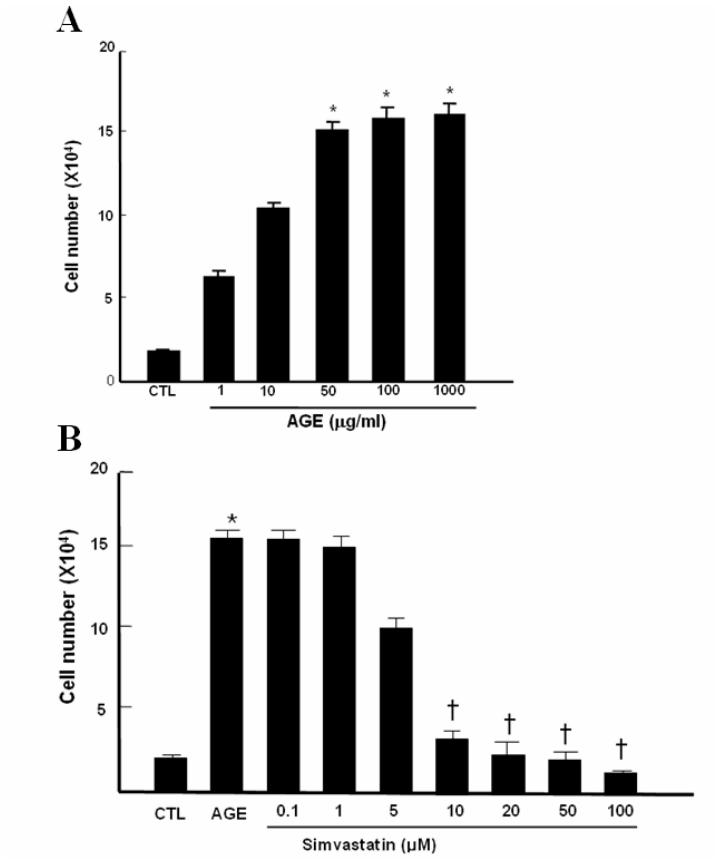

Figure 1. Effect of AGEs and HMG CoA reductase inhibitor on the proliferation of RASMCs. Cultured RASMC was co-incubated with prepared BSA-AGEs solution by each concentration of 1, 10, 50, 100 and 1000 μg/ml for 2 hours at 37℃ for 3h. HMG CoA reductase inhibitor was treated for inhibition experiment. The proliferation is increased with AGEs treatment dose-dependently (A) and decreased with HMG CoA reductase inhibitor dose-dependently (B). The proliferation of cell was determined by the MTT assay. Data are summary of three independent experiments and expressed as the mean ± SEM. *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* AGEs.

CTL, control ; AGE, Advanced glycation endproducts

2. Effect of AGEs and HMG CoA reductase inhibitor on the formation of intracellular ROS in RASMCs

Labeled rat aortic smooth muscle cells are examined using a luminescence spectrophotometer for oxidized dye. The quiescent cells are treated with AGEs for 3h before labeling with H_2DCFDA. As shown in Figure 2A, the degree of oxidative stress is increased with AGEs treatment dose-dependently and the increase was significant at 50 μg/ml of AGEs (1.5 ± 1.0 fold increase compared to control at 50 μg/ml of AGEs). HMG CoA reductase inhibitor inhibited the augmentation of oxidative stress induced by 50 μg/ml of AGEs (1.3 ± 0.9 fold reduction compared to AGEs-treated group at 10 μM of HMG CoA reductase inhibitor) (Figure 2B).

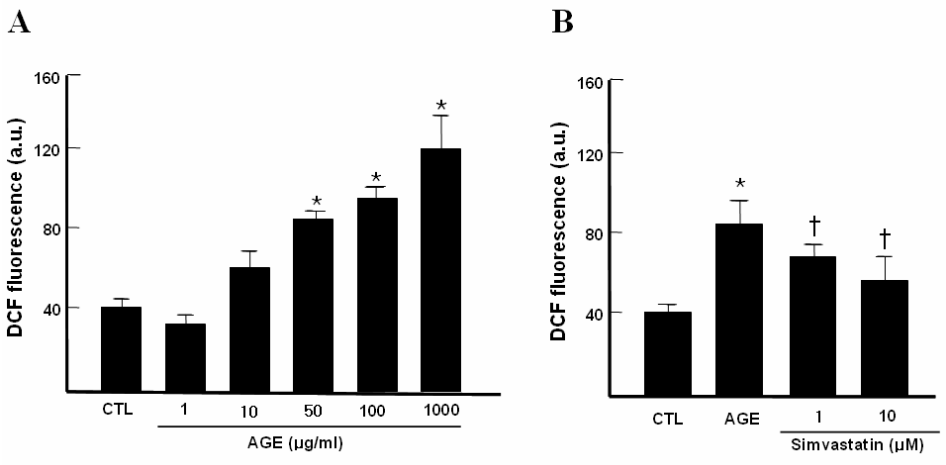

Figure 2. Effect of AGEs and HMG CoA reductase inhibitor on the formation of intracellular ROS in RASMCs. RASMCs were labeled with 2′,

7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. Labeled rat aortic smooth muscle cells were examined using a luminescence spectrophotometer for oxidized dye. The quiescent cells were treated with AGEs for 3 h before labeling with H_2DCFDA. Results shown are increased oxidative stress with AGEs treatment dose-dependently (A) and suppressive effect of HMG CoA reductase inhibitor on oxidative stress (B). Data are summary of three independent experiments and expressed as the mean ± SEM. *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* AGEs.
CTL, control ; AGE, Advanced glycation endproducts

3. Effect of HMG CoA reductase inhibitor on the activation of NF-κB in RASMCs

To confirm the inhibitory effect of HMG CoA reductase inhibitor on expression of NF-κB, the changes in NF-κB levels were estimated by immunoblot analysis. Cells were treated with 50 μg/ml of AGEs and 1, 10 μM of HMG CoA reductase inhibitor. AGEs (50 μg/ml) induced several fold increase in NF-κB (2.1 ± 1.3 fold increase compared to control) expression, but HMG CoA reductase inhibitor significantly inhibited AGEs-induced augmentation of NF-κB expression (2.4 ± 1.1 fold reduction compared to AGEs-treated group at 10 μM of HMG CoA reductase inhibitor) (Figure 3).

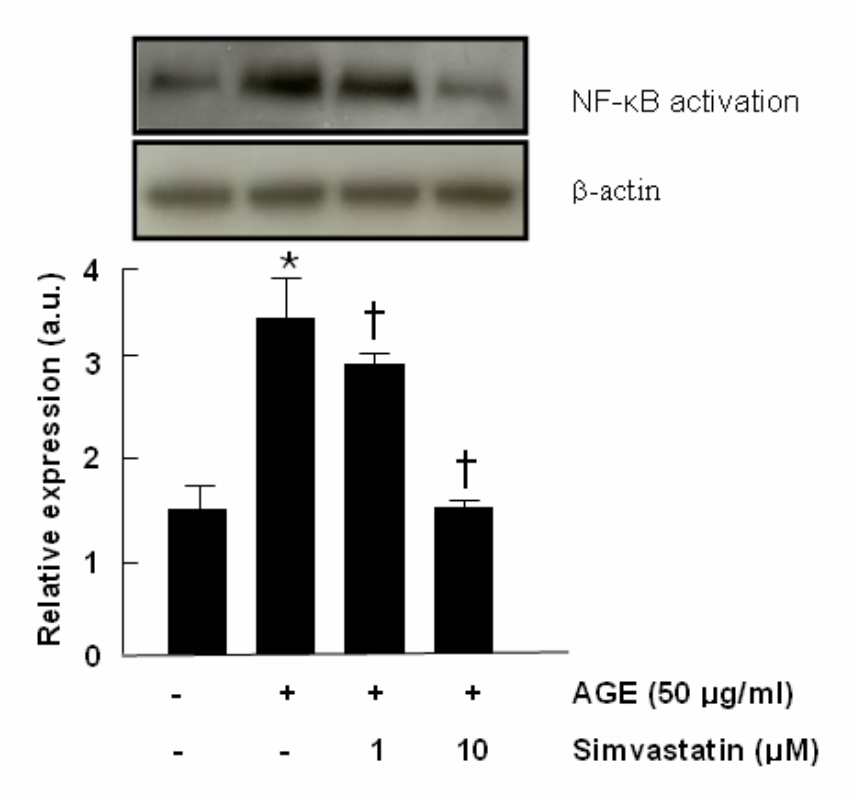

Figure 3. Effect of HMG CoA reductase inhibitor on the activation of NF-κB in RASMCs treated with AGEs. Western blot for β–actin is shown as a protein loading control. Cells were treated with 50 μg/ml of AGEs and 1, 10 μM of HMG CoA reductase inhibitor. Compared to cells treated with AGEs alone, cells treated with HMG CoA reductase inhibitor demonstrated significant inhibition of NF-κB expression. Symbols - and + indicate the absence and presence of AGEs or simvastatin, respectively. Data show a representative gel of NF-κB products and a bar graph of data quantitated from three to four experiments expressed as relative expression(a.u.) over respective control (mean ± SEM). *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* AGEs-treated.

AGE, Advanced glycation endproducts

4. Effect of AGEs and HMG CoA reductase inhibitor on the phosphorylation of MAPK in RASMCs

To confirm the inhibitory effect of HMG CoA reductase inhibitor on expression of MAPK, the changes in MAPK levels were estimated by immunoblot analysis. Cells were treated with 50 μg/ml of AGEs and 1, 10 μM of HMG CoA reductase inhibitor. AGEs (50 μg/ml) induced several fold increase in ERK and p38 (3.1 ± 1.2 fold ; 2.9 ± 1.0 fold increase compared to control) expression, but HMG CoA reductase inhibitor significantly inhibited AGEs-induced augmentation of ERK and p38 expression (2.0 ± 1.1 fold ; 2.3 ± 1.3 fold reduction compared to AGEs-treated group at 10 μM of HMG CoA reductase inhibitor) (Figure 4). This suggests that HMG CoA reductase inhibitor significantly inhibits activation of the MAPK increased with AGEs.

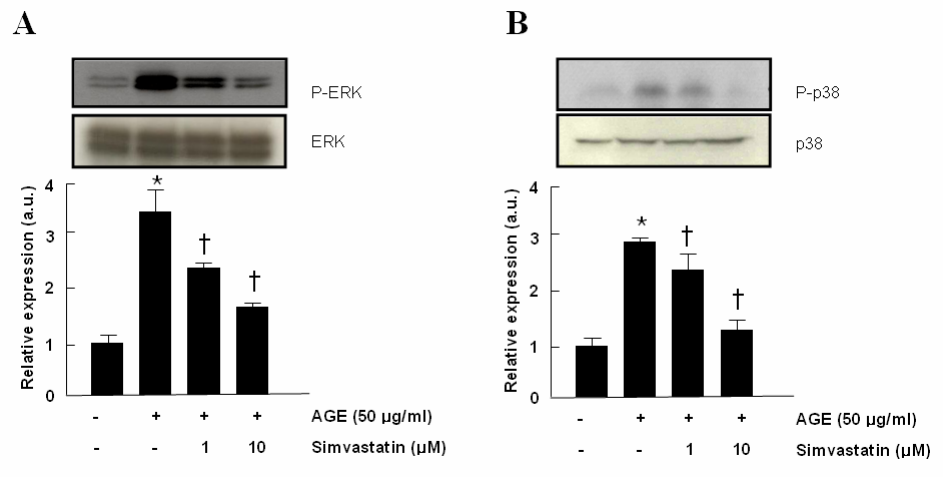

Figure 4. Effect of AGEs and HMG CoA reductase inhibitor on the phosphorylation of MAPK in RASMCs treated with AGEs. Western blot for

ERK and p38 in AGEs- or HMG CoA reductase inhibitor-treated RASMC. Cells were treated with 50 μg/ml of AGEs and 1, 10 μM of HMG CoA reductase inhibitor. The AGEs-treated one revealed increased activation of ERK (A) and p38 (B) and decreased activation with 1μM of HMG CoA reductase inhibitor. Symbols - and + indicate the absence and presence of AGEs or simvastatin, respectively. Data show a representative gel of ERK and p38 products and a bar graph of data quantitated from three to four experiments expressed as relative expression(a.u.) over respective control (mean ± SEM). *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* AGEs-treated.
AGE, Advanced glycation endproducts

5. Effect of HMG CoA reductase inhibitor and iRNA on the activation of MAPK in RASMCs

To determine whether AGEs is coupled to ERK activation via RAGE, siRNA for RAGE was pretreated. 50 μg/ml of AGEs increased phosphorylation of ERK 1.7 ± 0.8 fold compared to control and 10 μM of HMG CoA reductase inhibitor decreased phosphorylation of ERK (1.9 ± 1.2 fold reduction compared to AGEs-treated group). Also AGEs induced ERK activation was decreased to basal control level by RAGE inhibition. Furthermore, there was no difference of ERK activation between both and individual treatment of HMG CoA reductase inhibitor and siRNA for RAGE. These indicate that RAGE mediated the ERK activation by AGEs and HMG CoA reductase inhibitors blocked the process induced by RAGE-AGE interaction. (Figure 5).

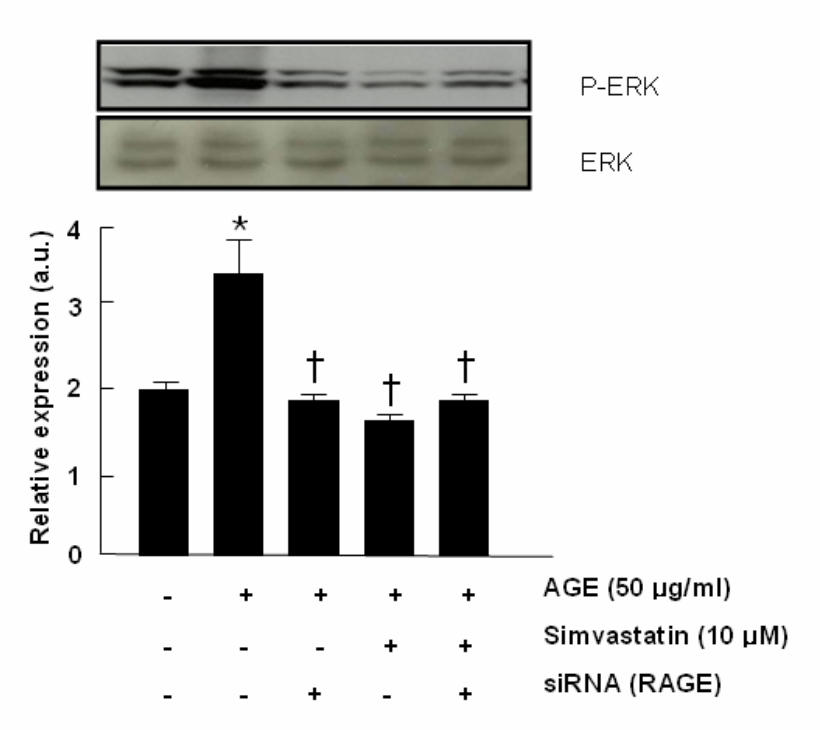

Figure 5. Effect of small interfering RNA molecules (siRNA) targeted at RAGE on the phosphorylation of MAPK in RASMCs treated with AGEs. Western blot for ERK in AGEs-, siRNA-treated or HMG CoA reductase inhibitor treated RASMC. For function-blocking experiments, small interfering RNA molecules (siRNA) targeted at RAGE mRNA was used. A 21-nt sequence for siRNA was derived from the rat RASMCs were transfected with si-RAGE duplexes. There was no difference of ERK activation between both and individual treatment of HMG CoA reductase inhibitor and siRNA for RAGE. These indicate that RAGE mediated the ERK activation by AGEs. Symbols - and + indicate the absence and presence of AGEs, simvastatin or iRNA, respectively. Data show a representative gel of ERK products and a bar graph of data quantitated from three to four experiments expressed as relative expression(a.u.) over respective control (mean ± SEM). *, $p < 0.05$ *versus*

control; †, $p < 0.05$ *versus* AGEs-treated.
AGE, Advanced glycation endproducts
siRNA, small interfering RNA

6. Effect of HMG CoA reductase inhibitor on the suppression of c-jun and COX-2 mRNA expression in RASMCs.

The inhibition of expression of the c-jun and COX-2 at the mRNA level was demonstrated by RT-PCR. The treatment of RASMCs with AGEs resulted in increased expression levels of c-jun and COX-2, which was reduced by pretreatment of HMG CoA reductase inhibitor (2.9 ± 1.1 fold ; 2.1 ± 0.9 fold reduction compared to AGEs-treated group at 10 μM of HMG CoA reductase inhibitor) (Figure 6). The results indicated that HMG CoA reductase inhibitor has an inhibitory effect on the protein expression levels of c-jun and COX-2.

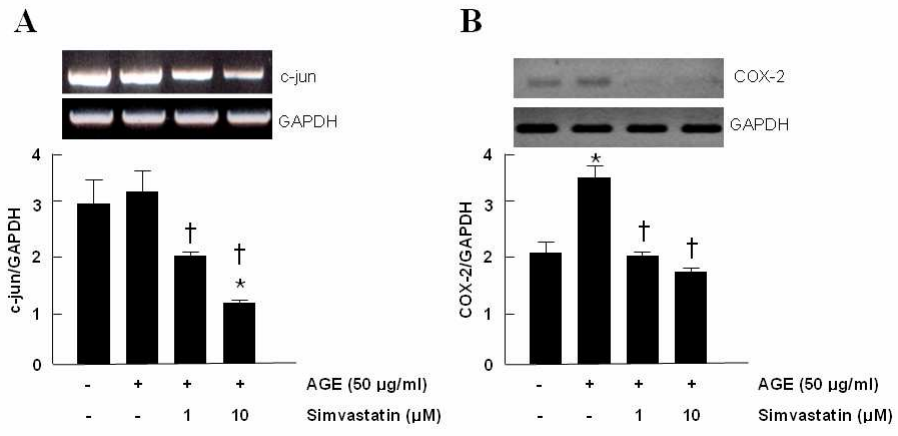

Figure 6. Effect of HMG CoA reductase inhibitor on the suppression of

protooncogene, c-jun and COX-2 mRNA expression induced by AGEs. Cells were treated with 50 μg/ml of AGEs and 1, 10 μM of HMG CoA reductase inhibitor. The expression levels of various proteins were analyzed by reverse transcription polymerase chain reaction (RT-PCR). The expressions of genes were analyzed by RT-PCR using GAPDH as an internal standard. The expression of c-jun (A) and COX-2 (B) revealed increase with AGEs and decrease with HMG CoA reductase inhibitor dose-dependently. Symbols - and + indicate the absence and presence of AGEs or simvastatin, respectively. Data show a representative gel of c-jun and COX-2 products and a bar graph of data quantitated from three to four experiments expressed as ratio of protein to its respective GAPDH signal intensity (mean ± SEM). *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* AGEs-treated.

AGE, Advanced glycation endproducts

7. Impact of HMG CoA reductase inhibitor on neointimal hyperplasia and the expression of COX-2 in carotid artery of diabetic rat

Compared with control, neointimal formation of carotid artery of diabetic model showed an increased proliferation. However, diabetic model treated with HMG CoA reductase inhibitor showed more reduced neointimal formation than not treated (1.9 ± 0.1 fold reduction compared to control) (Figure 7A-C and bar graph).

COX-2 staining was more abundant in lesions of carotid artery from AGEs-treated rat but markedly decreased after diabetic model treated with HMG CoA reductase inhibitor (Figure 7D-F).

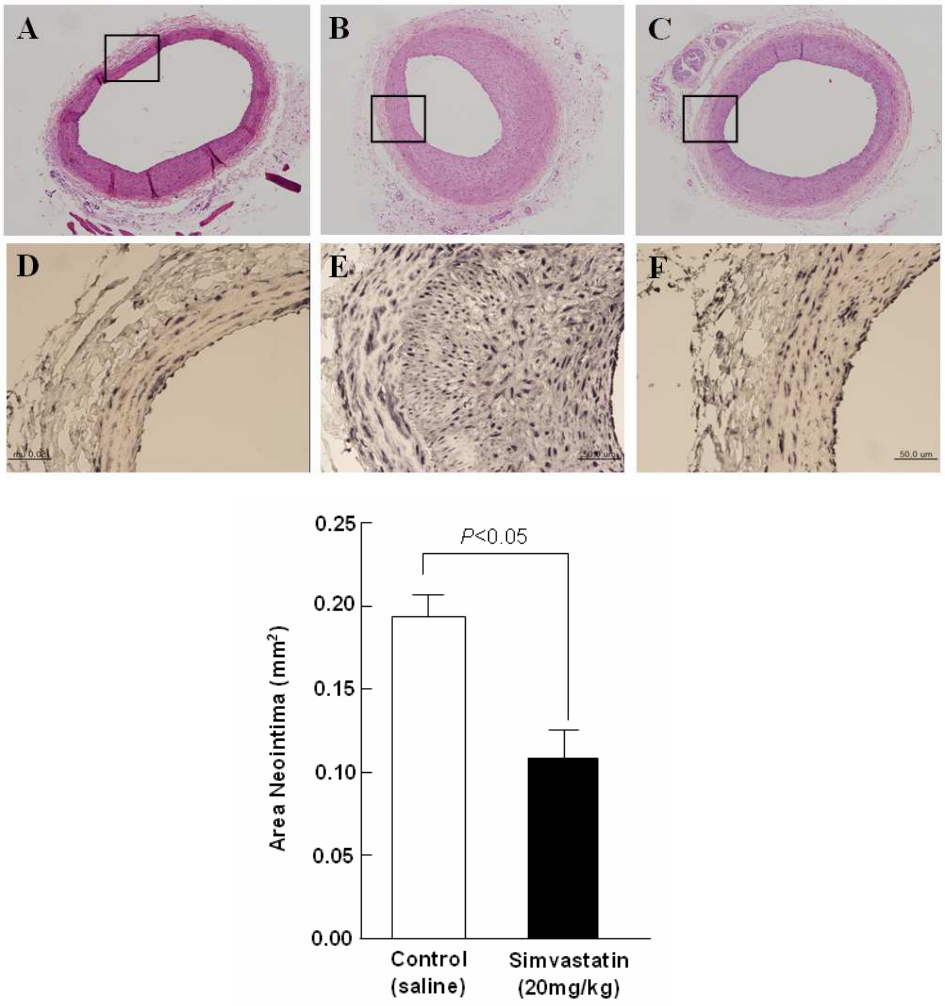

Figure 7. H-E staininig and immunohistochemistry for COX-2 of sections of carotid artery from control, AGEs-treated rat and HMG CoA reductase inhibitor-treated rat. The treated model with HMG CoA reductase inhibitor (C) showed similar intimal thickness with control (A) but more reduced neointimal hypertrophy than not treated one (B) (X100, H-E staining). COX-2 staining was more abundant in lesions from AGEs-treated model (E) than control (D) or HMG CoA reductase inhibitor treated one (F). Immunohistochemical analysis of carotid artery stained (X200) with specific

antibodies for COX-2.

8. Expression of RAGE in human atherosclerotic plaque of common carotid artery

Human carotid endarterectomy specimens were obtained from diabetic patients undergone carotid endarterectomy. Carotid atheroma specimens showed a thickened intima associated to an area of necrotic core and lipid-laden atheroma in all cases. RAGE was stained as dark brown within atheromatous plaques. RAGE stained cells were distributed mainly in the base of atherosclerotic plaque, in the medio-intimal junction area and its immunoreactivity was colocalized with macrophage, mononuclear cells, and smooth muscle cells (Figure 8).

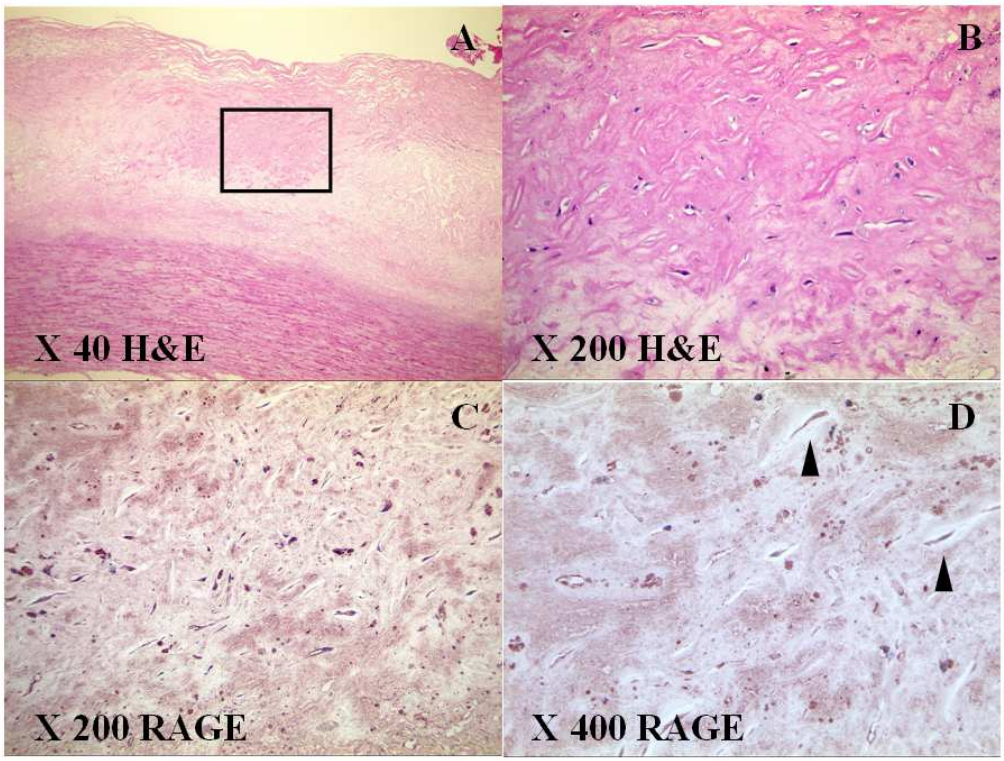

Figure 8. H-E staining and immunohistochemistry for RAGE in human carotid atheroma. RAGE stained cells were distributed mainly in the base of atherosclerotic plaque, in the medio-intimal junction area and its immunoreactivity was colocalized with macrophage, mononuclear cells, and smooth muscle cells. RAGE was stained as dark brown within atheromatous plaques (arrow heads).

IV. DISCUSSION

Recent studies have demonstrated that AGEs and its receptor/ligand interaction play a key role in neointimal formation after vascular injury irrespective of diabetes status[5-7] and generation of ROS that can function as

signal transduction molecules and play an important role in the inflammatory process and atherosclerosis[9,21]. In rats, HMG CoA reductase inhibitors reduces ROS production[17] and prevents cardiac myocyte hypertrophy[18]. It revealed that HMG CoA reductase inhibitors inhibit p38 MAPK and consequently inhibit the membrane translocation of the small G protein family[19]. However, little is known about the molecular mechanism of these drugs as antioxidants. In the present study we provide evidence for the critical involvement of HMG CoA reductase inhibitor in the process of AGEs-induced intracellular ROS of VSMCs. Furthermore, the present findings are the first to our knowledge to demonstrate a mechanism of inhibitory effect of HMG CoA reductase inhibitor on the process of developing diabetic vasculopathy in view of AGEs-induced intracellular oxidative stress and consequent cellular signaling.

Cardiovascular disease is increased in individuals with Diabetes Mellitus (DM). Type 2 diabetes patients without a prior MI have a similar risk for "coronary artery–related events" as non-diabetic individuals who have had a prior myocardial infarction. The increase in cardiovascular morbidity and mortality appears to relate to the synergism of hyperglycemia with other cardiovascular risk factors. For example, after controlling for all known cardiovascular risk factors, type 2 DM increases the cardiovascular death rate twofold in men and fourfold in women. Recent work has focused on the role of AGEs and RAGE in macro- and microvascular complications in DM. There are several potential mechanisms by which AGEs may be involved in macrovascular disease[23,24]. These include activation of monocytes, production of cytokines and growth factors, impairment of endothelial function, modification of LDL, and depletion of nitric oxide. Binding of AGEs to its receptor, RAGE on cell surfaces, including endothelial cells and VSMCs, induces an intracellular oxidative stress response characterized by increased activation of transcription factors, e.g., NFκB[25]. Oxidative stress of the vascular wall is a hallmark of atherosclerosis[26] and consequent activation of NFκB and

MAPK system may be the possible mechanism of AGEs induced vasculopathy[27]. More recently, the role of ROS as a signal molecule has gained increasing attention. The cytotoxicity of ROS may be associated with the ability of ROS to signal distinct pathways, such as the NFκB pathway, to induce pathology. NFκB comprises a family of transcription factors that are involved in regulating a large number of genes related to immune function, inflammation, apoptosis and cell proliferation. So it is a key mediator of a variety of cellular responses – (1) Immune and inflammatory response, (2) cell proliferation and survival. Many chronic disease states have been associated with aberrant activation of NFκB, and several therapeutic strategies targeting NFκB activation have been considered for the treatment of inflammation and cancer[28]. And there are studies demonstrating that treatment of monocytes with *in vitro* prepared AGEs or the specific RAGE ligand can significantly increase COX-2 mRNA and its pro-inflammatory products in THP-1 human monocytes[31]. COX-2 and its pro-inflammatory products have been implicated in the pathogenesis of atherosclerosis, and it is also induced by oxidized lipids[34-37]. COX-2 was shown to promote early atherosclerotic lesion formation in low density lipoprotein receptor-deficient mice[35]. Another studies demonstrated that interaction of RAGE and its ligand is closely correlated with cell migration and invasion induced by intracellular signaling pathways, including GTPases, Cdc42, Rac, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)-1/2, p38, c-Jun N-terminal kinase (JNK) and NF-κB[32,33].

HMG CoA reductase inhibitors, statins are effective lipid-lowering agents. Recent experimental studies suggested that the beneficial effects of HMG CoA reductase inhibitor in patients at risk for cardiovascular disease are not only a result of an improved lipid profile but also mediated by direct vasculoprotective actions[26,29]. Another accumulating evidence suggests that HMG CoA reductase inhibitors have anti-inflammatory and endothelial cell protective actions that are independent of their cholesterol lowering effect[30]. And moreover, in recent

study, RAGE suppression by simvastatin is emphasized to be largely dependent on the reduction of AGEs generation by myeloperoxidase[27]. Antioxidant effect of HMG CoA reductase inhibitors is especially important in the aspects of diabetic complications such as macrovascular or microvascular pathology. AGEs induced smooth muscle cell proliferation and oxidative stress formation is emerging as one of the important mechanism of diabetic vasculopathy, but little is known about pleiotrophic action of HMG CoA reductase inhibitors on AGEs. There are many factors of inducing ROS such as PDGF, thrombin, phenylephrine, Angiotensin II, TNF, oxidized LDL, hyperglycemia, VEGF, and the interaction between AGE and RAGE evokes many events like above. ROS itself is known to be induced by the activation of NADPH oxidase from the reaction between AGEs and RAGE, therefore we investigated to determine whether HMG CoA reductase inhibitors blocks vascular smooth muscle cell proliferation through inhibition of the ROS-induced proliferation and inflammation pathway. This hypothesis was derived from the fact that the AGE-RAGE interaction may be the upstream mechanism that increases the formation of ROS and resultant vascular smooth muscle cell proliferation. Therefore, we designed a schema showing the possible cascade of mechanism of diabetic vasculopathy induced with AGEs and the inhibitory action of HMG CoA reductase inhibitors in this process (Figure 9).

There are many reports showing the proliferrative and proinflammtory effect of reaction of RAGE and its ligands in view of the pathophysiology of cancer progression, inflammatory diseases, diabetes and Alzheimer's disease. Activation of RAGE is known to stimulate ERK and NFκB activity and to increase cell proliferation, survival, and motility[38-40]. HMG CoA reductase inhibitors are reported to have anti-inflammatory effect on cardiovascular disease[19] and our data showed increased cell proliferation and inflammatory reactions with AGEs treatment in dose dependent manners and reduced cell proliferation, inflammatory cellular signaling and protein expression of

RASMC with HMG CoA reductase inhibitors. To clarify the inflammatory and

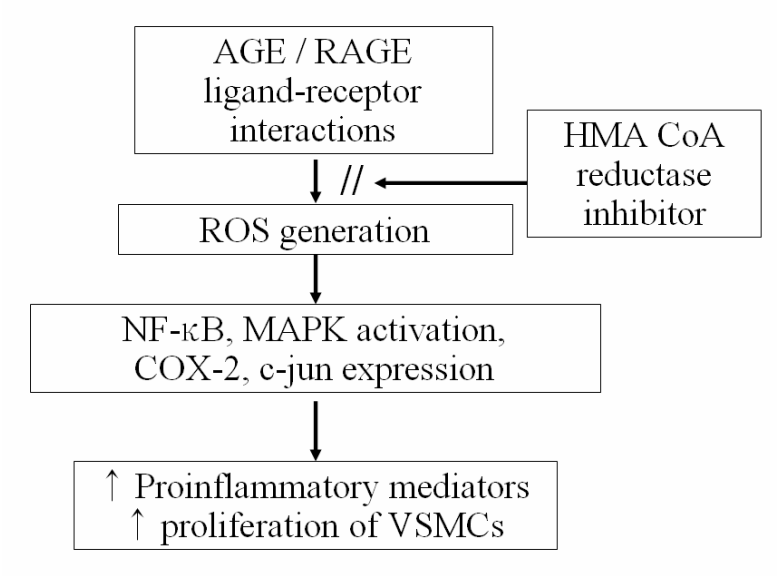

Figure 9. Schema of possible mechanism of inhibitory effect of HMG CoA reductase inhibitors on AGEs-induced vasculopathy. In the cascade of AGEs induced ROS formation, inflammatory and proliferative cellular signaling, HMG CoA reductase inhibitors may act as blocker of the process from AGE-RAGE interaction to ROS generation.

proliferative action, we used proteins of NFκB, p38, ERK, COX-2 and c-jun as important factors in inflammatory cascade. Cuccurullo C. et al. characterized the effect of simvastatin on the expression of RAGE and RAGE-dependent plaque-destabilizing genes such as COX-2 in human atherosclerotic plaques[27]. They hypothesized that simvastatin might inhibit plaque RAGE expression by

decreasing myeloperoxidase (MPO)-dependent AGE generation in turn contribute to plaque stabilization by inhibiting the biosynthesis of PGE2-dependent metalloproteinase (MMPs), responsible for plaque rupture[40]. Our data didn't show the difference of AGE or RAGE expression in control and those treated with HMG CoA reductase inhibitors. It can be a limitation of our study, but our hypothesis is that HMG CoA reductase inhibitors may block the process from RAGE-AGE interaction to ROS generation and the data of siRNA supported the hypothesis.

To determine whether HMG CoA reductase inhibitors suppressed cellular signaling induced by RAGE-AGE interaction, we examined the effect of small interfering RNA molecules (siRNA) targeted at RAGE mRNA, for function-blocking experiments. ERK activation was increased compared to control with AGEs and decreased activation was examined with HMG CoA reductase inhibitor. Also AGEs induced ERK activation was decreased to basal control level by RAGE inhibition. Furthermore, there was no difference of ERK activation between both and individual treatment of HMG CoA reductase inhibitor and siRNA for RAGE. These indicate that RAGE mediated the ERK activation by AGEs and HMG CoA reductase inhibitors blocked the process induced by RAGE-AGE interaction.

Recent experimental studies emphasize the antioxidative effects of HMG CoA reductase inhibitors and suggest that HMG CoA reductase inhibitors reduce reactive oxygen species in endothelial cells by S-nitrosylation of thioredoxin[13,14,22]. Recently, several reports have suggested that HMG CoA reductase inhibitors could act as antioxidants in experimental atherosclerosis in rabbits[15], inhibiting the oxidation of LDL by activated monocyte-derived macrophages[16]. In rats, HMG CoA reductase inhibitors reduces ROS production[17] and prevents cardiac myocyte hypertrophy[18]. The ROS formation in our data revealed increased with AGEs stimulation and reduced with HMG CoA reductase inhibitors treatment in dose dependent manners like other

studies.

Many other articles revealed anti-atherogenic effect of HMG CoA reductase inhibitors by using vascular injury model in vivo study[41]. In this study, the same data was shown as resultant product of anti-inflammatory, anti-proliferative and anti-oxidative action of HMG CoA reductase inhibitors.

In the present study HMG CoA reductase inhibitors reduced the AGE-RAGE interaction-induced increased intracellular oxidative stress (ROS) and ROS-induced cellular signaling (activation of NF-κB, MAPK and expression of COX-2, c-jun) in vascular smooth muscle cells. Consequently it brought the suppression of neointimal hypertrophy and expression of COX-2 in carotid artery of diabetic rat model. Therefore, this study shows the possible mechanism of action of HMG CoA reductase inhibitors in the antiproliferative effect on VSMCs of diabetic model. However, it remains possible that HMG CoA reductase inhibitors also have many additional effects on diabetic vasculopathy and further studies will be necessary to fully understand actions of HMG CoA reductase inhibitors.

In suggesting clinical implication, the dose of intervention is important in clinical area. We used 20 mg/kg of high dose in rat, but human dose is lower. So the limitation can be that we did not suggest the clinical dose of human. Because the effect of various chemical structures of HMG CoA reductase inhibitors on ROS formation can be different and we used only one kind of HMG CoA reductase inhibitor, so it can be another limitation of this study.

V. CONCLUSION

Antioxidant effect of HMG CoA reductase inhibitors is especially important in the aspects of diabetic complications such as macrovascular or microvascular

pathology. This study demonstrated the possible mechanism of action of HMG CoA reductase inhibitors in the antiproliferative effect on vascular smooth muscle cell of diabetic model. Conclusively, HMG CoA reductase inhibitor is expected to play a key role in treatment of AGEs-induced diabetic vasculopathy.

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ABSTRACT (In Korean)

당뇨성 혈관병증에서 혈관 평활근 세포에 대한 Advanced Glycation Endproducts (AGEs)에 의한 산화 스트레스에 대한 HMG CoA Reductase Inhibitor의 역할

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최근 HMG CoA reductase inhibitor의 항산화 작용은 그 다양한 효과 중의 한가지로 알려져 있으며 특히 당뇨성 혈관병리에 있어서 매우 중요하다. Advanced glycation endproducts (AGEs)에 의한 reactive oxygen species (ROS)의 발생과 평활근 세포 증식이 당뇨성 혈관병증의 중요한 기전으로 부각되고 있으나 AGEs에 대한 HMG CoA reductase inhibitor의 항산화 작용에 대해서는 아직 자세히 알려진 바가 없다. 이에 본 연구에서는 HMG CoA reductase inhibitor가 AGEs로 인해 증가된 혈관 평활근 세포내 산화 스트레스를 감소시킬 것이라는 가설을 세우고 ROS로 인해 유발된 세포내 전달 기전에 영향을 미치는 HMG CoA reductase inhibitor의 역할에 대한 가능한 기전을 분석하고자 하였다. AGEs를 처리하였을 때 용량에 비례하여 백서의

혈관 평활근 세포의 증식과 ROS의 증가를 보였으며 NFκB, phosphorylated ERKs, phosphorylated p38, COX-2과 c-jun의 발현이 증가되었다. 대조군(HMG CoA reductase inhibitor 처리하지 않은)과 비교해서 HMG CoA reductase inhibitor를 처리한 군은 AGEs로 인한 혈관 평활근 세포의 증식과 ROS 생성, 그리고 NFκB, phosphorylated ERKs, phosphorylated p38, COX-2과 c-jun의 발현이 용량에 비례하여 억제되었다. 풍선으로 손상을 준 후 그렇지 않은 군보다 백서의 총경동맥 신생 내막의 형성이 증가되었으나 HMG CoA reductase inhibitors로 치료을 한 군에서 신생 내막의 형성이 억제된 것을 볼 수 있었다. RAGE (AGEs에 대한 수용체)에 대한 siRNA를 사용했을 때 HMG CoA reductase inhibitor를 사용한 군과 ERK의 발현에는 차이가 없었다. 본 연구에서 AGEs는 혈관 평활근 세포 증식과 ROS 증가에 중요한 역할을 하는 것을 보여주고 있으며 HMG CoA reductase inhibitor는 AGEs로 인한 혈관 평활근 세포의 증식, ROS 생성 그리고 MAPK의 활성도를 억제시키는 것을 알 수 있다. 결론적으로, ROS 생성과 MAPK의 활성화는 AGEs로 인한 당뇨성 혈관병리의 가능한 기전으로 HMG CoA reductase inhibitor는 이러한 AGEs로 인한 당뇨성 동맥경화의 치료에 있어서 중요한 역할을 할 것이라는 결론을 내릴 수 있다.

핵심되는 말 : AGEs, ROS, 혈관 평활근 세포 증식, HMG CoA reductase inhibitor, NFκB, ERK, p38, c-jun, COX-2