Mechanisms Involved in the Stimulatory Effect of Advanced Glycation End Products on Growth of Rat Aortic Smooth Muscle Cells

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Hyperglycemia is an important cause of accelerated atherosclerosis in diabetic patients. We examined the effect of hyperglycemia and advanced glycation end products (AGE) on proliferation of rat aortic smooth muscle cells (SMC) in culture; in vivo, this event is believed to contribute importantly to atherogenesis in diabetes mellitus. Glucose itself dose-dependently inhibited thymidine uptake by SMC, but AGE increased thymidine uptake, suggesting that SMC proliferation is accelerated by AGE. To examine possible mechanisms for this effect, we studied nuclear factor-kappa B (NF-κB) activation and the tyrosine phosphorylation pathway; AGE stimulated NF-κB activity, but phosphorylation of the platelet-derived growth factor (PDGF) receptor was unchanged. In Chinese hamster ovary (CHO) cells overexpressing galectin-3, an AGE receptor related to atherosclerosis, AGE increased thymidine uptake. This suggests SMC proliferation is enhanced by AGE via galectin-3. As pathways involving AGE–galectin-3 interaction thus may be involved in macroangiopathy, AGE appears to be important to the role of SMC in accelerated atherosclerosis associated with diabetes mellitus.

Microangiopathy and cardiovascular disease, also known as macroangiopathy, are the leading causes of mortality in diabetic patients. The primary cause of cardiovascular disease is atherosclerosis. Vascular smooth muscle cells (SMC) play a crucial role in atherosclerosis, as they proliferate, migrate, and express genes encoding inducible growth factors involved in extracellular matrix remodeling. Hyperglycemia is considered important in the development of vascular complications in diabetic patients.[1] However, the mechanisms accelerating atherosclerotic changes induced by hyperglycemia in diabetes remain unclear.

Hyperglycemia accelerates nonenzymatic glycation of protein (Maillard reaction); a complex series of rearrangements and oxidative reactions leads to formation of advanced glycation end products (AGE). In diabetes, AGE increase in plasma, and deposit in vessels, kidney, cardiac muscle, crystalline lens, and atherosclerotic lesions.[2,3] AGE are thought to be important in diabetic angiopathy.[4] Some AGE receptors have been identified, such as the receptor for AGE (RAGE),[5] macrophage scavenger receptor (MSR),[6] CD36,[7] and galectin-3.[8] Galectin-3, a soluble β-galactoside–binding lectin, has various biologic functions and was reported to be linked to atherosclerosis.[9,10]

SMC are central in atherosclerotic lesion formation, showing not only increased migration but also accelerated proliferation.[11] To clarify the respective roles of AGE and high plasma glucose per se in acceleration of atherosclerosis in diabetes, we examined the effects of high concentrations of glucose or AGE on SMC proliferation and possible underlying mechanisms including nuclear factor-kappa B (NF-κB) as well as its receptor as expressed by galectin-3–transfected Chinese hamster ovary (CHO) cells.

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MATERIALS AND METHODS

Culture of the Cells

Rat aortic SMC were obtained from Wistar Kyoto rats (Charles River, Yokohama, Japan) using an explant method. The SMC were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Rockville, MD) containing penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (FBS).

Galectin-3–overexpressing CHO cells were maintained in DMEM containing penicillin (100 U/mL), streptomycin (100 mg/mL), geneticin (200 μg/mL), and 10% FBS.[12]

Experiments were performed in compliance with the Principles of Laboratory Animal Care (US NIH publication no. 85-23, revised 1985).

Preparation of AGE–Bovine Serum Albumin

AGE–bovine serum albumin (AGE-BSA) was prepared by incubating BSA (fraction V; WAKO, Tokyo, Japan) in phosphate-buffered saline with 0.7 mol/L glucose for 6 months at 37°C as reported previously.[13]

Effects of Glucose Concentration on Cell Numbers

SMC (passage 5 to 10) were distributed in 6-well plates and grown for 5 days in DMEM containing 5.6 mmol/L, 10.5 mmol/L, 15.3 mmol/L, 20.1 mmol/L, or 25 mmol/L glucose. After incubation for 24 hours with DMEM containing 0.2% BSA and 0.4% FBS, and the various concentrations of glucose, cells in each well were counted.

Measurement of Tritium-Thymidine Uptake

Eighty percent to 90% confluent cells were made quiescent for 24 hours using medium containing 0.2% BSA and 0.4% FBS before each stimulation. Tritium-thymidine (4 μCi/well) was added to the cells during the last 6 hours of each stimulation. Cells were washed with phosphate-buffered saline and fixed with 5% trichloroaetic acid. The precipitate was dissolved in 1 mL of KOH solution (0.5 mol/L) and radioactivity was measured in a liquid scintillation counter.

Protein Extraction From Cultured Rat Aortic SMC

Rat aortic SMC were homogenized in 50 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) at pH 7.6 containing

1,000 U/mL aprotinin, 0.002% phenyl-methylsulfonyl fluoride, 20 μg/mL leupeptin, 0.1 mmol/L benzamidine, 1 mmol/L dithiothreitol (DTT), and 2 mmol/L EDTA, 4 mmol/L orthvanadate, and 1% Triton X-100 at 4°C. Samples then were centrifuged at 15,000 rpm, and the final supernatant was stored at −80°C.

Tyrosine Phosphorylation of the Platelet-Derived Growth Factor-β Receptor

SMC were incubated with 10 μg/mL AGE-BSA or BSA alone as a control for 48 hours, and then stimulated with 10 ng/mL platelet-derived growth factor (PDGF; Cell Biology Boehringer Mannheim, Mannheim, Germany) for 5 minutes. A 1-μg quantity of anti- PDGFβ receptor antibody (Transduction Laboratories, Lexington, KY) was added to 100 μg of cell lysates and incubated at 4°C for 1 hour. Protein A (Pierce, Rockford, IL) was added to this reaction mixture and rotated gently at 4°C for 1 hour. Agarose beads were centrifuged at 15,000 rpm for 5 minutes, washed 2 times with 50 mmol/L HEPES, and collected. The pellets were used for assay of Western blot analysis. The same amount of each immunoprecipitated sample was fractionated by electrophoresis in 10% acrylamide resolving gel containing 10% (vol/vol) glycerol, and protein bands were transferred to a nitrocellulose filter. The filter was incubated with 1 μg/mL of anti–phosphotyrosine antibody (ICN Biomedicals, Aurora, OH) at 4°C overnight, washed, and incubated with 1 μg/mL second antibody (anti-mouse IgG labeled with peroxidase; Amersham Biosciences, Piscataway, NJ) for 45 minutes at room temperature. The filter was washed, and bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

Western Analysis of Galectin-3

Samples were fractionated by electrophoresis in 10% acrylamide resolving gel containing 10% (vol/vol) glycerol and transferred to a nitrocellulose filter. The filter was incubated overnight at 4°C with 1 μg/mL of galectin-3 antibody (Novocastra Laboratories, Newcastle, England) and then washed. Next the filter was incubated with 1 μg/mL of secondary antibody (anti-mouse IgG labeled with peroxidase) for 45 minutes at room temperature, washed, and visualized using an ECL kit.

Gel Shift Mobility Assay of NF-κB

Nuclear extracts were prepared according to the method of Marui et al.[14] NF-κB consensus oligonucleotides were obtained from Promega (Madison, WI). After annealing, double-stranded DNA was labeled with [γ-^{32}P]adenosine triphosphate (ATP) using T4 kinase and purified on a Sephadex G-25 column (Amersham Biosciences). Nuclear protein was incubated with [γ-^{32}P] ATP-labeled oligonucleotide probe at room temperature for 20 minutes in binding buffer containing 4% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), and 0.05 mg/mL poly (dI-dC). Competition experiments were performed by incubation of nuclear protein with unlabeled oligonucleotides. Protein-DNA complexes were resolved on 4% native polyacrylamide gels using 1x Tris-glycine buffer. Gels were dried and allowed to expose to radiographic film overnight at −70°C.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from cultured rat SMC by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method.[15] Reverse-transcription polymerase chain reaction (RT-PCR) was performed with 1 μg of total RNA using a 1-step RT-PCR kit (Quiage, Hilden, Germany). After reverse transcription (50°C for 30 minutes), the reaction mixture was incubated at 95°C for 15 minutes to inactivate reverse transcriptase and activate HotStar Taq DNA polymerase. Complementary DNA was amplified using primer sequences as follows: for galectin-3, upstream primer, 5′-AATGGCAGACGGCTTCTCACTT-3′, downstream primer, 5′-TAACACACAGGGCAGTTCTGGT-3′; according to a previous report.[16] PCR was performed in a Gene Amp PCR system 9600 (Perkin Elmer, Norwalk, CT). PCR conditions included initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 10 minutes. PCR products were electrophoresed in a 1% agarose gel and visualized with ethidium bromide under ultraviolet light. PCR products were sequenced using a Thermo Sequenase Cy 5.5 dye terminator cycle sequencing kit (Amersham Biosciences).

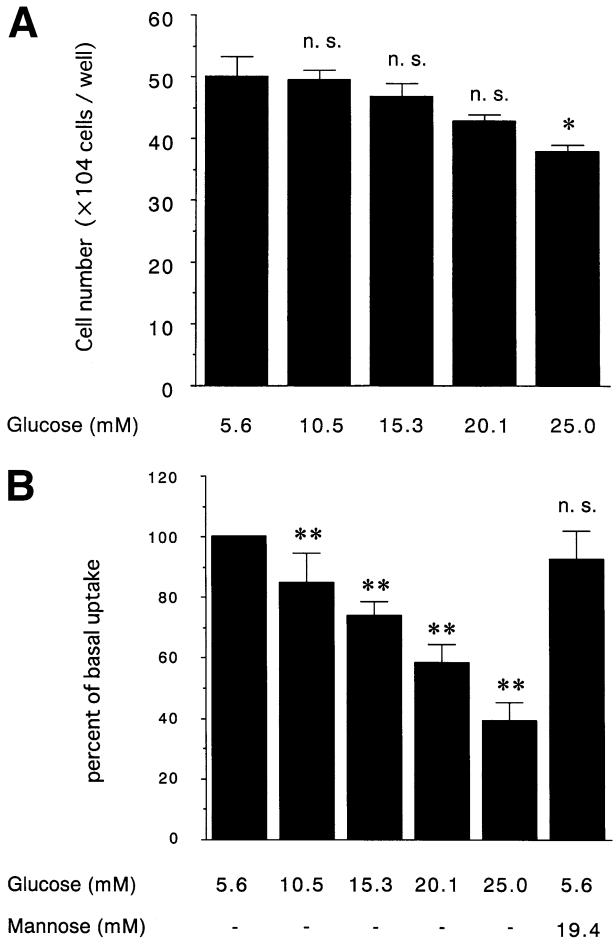

Fig 1. (A) Effect of glucose concentration on the cell numbers of SMC. The results represent the mean ± SEM (*P < .05) (n = 8). (B) Effects of glucose concentration on tritium-thymidine uptake of rat aortic SMC. Cells were cultured for 5 days in DMEM containing different concentrations of glucose (5.6, 10.5, 15.3, 20.1, or 25 mmol/L). Values represent percent of basal uptake. Data are shown as means ± SEM (P < .01, *P < .05; n = 4).**

Statistical Analysis

Values are expressed as means ± SEM. Sets of data were compared with 2-tailed unpaired Student's t test or the Mann-Whitney U test.

RESULTS

Effects of Glucose Concentration on Cell Numbers

As shown in Fig 1A, incubation with 25 mmol/L glucose

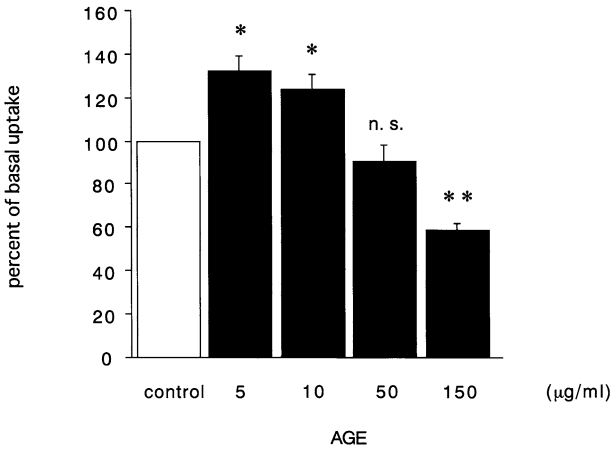

Fig 2. Effect of AGE-BSA on tritium-thymidine uptake in cultured rat aortic SMC. SMC were grown in DMEM containing 5.6 mmol/L glucose and 10% FBS for 5 days and incubated with different concentrations (5 to 150 μg/mL) of AGE-BSA for 48 hours in DMEM containing 10% FBS. As controls for AGE-BSA, identical concentrations of BSA were used. Values represent percent of basal uptake. Data are shown as means ± SEM ($P < .01$, *$P < .05$; n = 8).**

significantly decreased numbers of rat aortic SMC compared with 5.6 mmol/L glucose ($37.6 \pm 1.33 \times 10^4$ *v* $49.5 \pm 3.00 \times 10^4$/well; $P < .05$; n = 8). Other concentrations of glucose did not have significant effects.

Effects of Glucose Concentration on Tritium-Thymidine Uptake

High glucose concentration dose-dependently inhibited FBS-stimulated tritium-thymidine uptake by rat aortic SMC compared with 5.6 mmol/L glucose (Fig 1B). Exposure to 10.5, 15.3, 20.1, and 25 mmol/L glucose significantly and dose-dependently decreased tritium-thymidine uptake to 85% ± 9.6% ($P < .01$), 74.1% ± 4.5% ($P < .01$), 58.5% ± 6.2% ($P < .01$), and 39% ± 6.2% ($P < .01$) of baseline, respectively. To adjust for the osmotic effect of 25 mmol/L glucose, mannose was used as a control; mannose did not significantly change uptake compared with 5.6 mmol/L glucose.

Effect of AGE-BSA on Tritium-Thymidine Uptake

Figure 2 shows the effect of various concentrations of AGE-BSA for 48 hours on thymidine uptake in rat aortic SMC. Incubation with 5 and 10 μg/mL AGE-BSA increased thymidine uptake to 1.2 to 1.3 times that with control BSA ($P < .05$; n = 8). AGE-BSA at 50 μg/mL had no effect on thymidine uptake, while AGE-BSA at 150 μg/mL inhibited thymidine uptake.

Tyrosine Phosphorylation of PDGFβ Receptor

Incubation with 10 μg/mL AGE-BSA for 48 hours did not change tyrosine phosphorylation of the PDGFβ receptor with 10 ng/mL PDGF stimulation, as illustrated in Fig 3.

Gel Shift Mobility Assay

Stimulation with 10 μg/mL AGE-BSA increased NF-κB activity in rat aortic SMC (Fig 4). Two days (48 hours) of stimulation activated NF-κB more than did 1 hour of stimulation. This activation of NF-κB was inhibited by incubation with unlabeled oligonuleotides

Expression of Receptor of AGE on Rat SMC

In cultured rat SMC, expression of galectin-3 was analyzed by RT-PCR. The expected sizes of amplified partial rat galectin-3 DNAs were 832 base pairs, respectively (Fig 5A). These DNA fragments were confirmed by sequencing. In Western blotting, expression of galectin-3 was detected in protein level at 31 kd (Fig 5B).

Effect of Overexpression of Galectin-3 on Tritium-Thymidine Uptake

In wild-type CHO cells, 10 μg/mL AGE-BSA stimulation has no effect on thymidine uptake. In galectin-3–transfected cells, thymidine uptake was increased with 10 μg/mL AGE-BSA stimulation compared with control BSA ($P < .01$; n = 8) (Fig 6).

DISCUSSION

To clarify the mechanisms of accelerated atherosclerosis in diabetes, we examined the effects of high-glucose or high-AGE environment on growth of cultured SMC. SMC are believed to migrate within the arterial wall from the media to the intima, where their proliferation contributes to intimal thickening as a component of atherosclerosis.[11] Thymidine uptake by rat aortic SMC cultured in high glucose concentrations revealed a dose-dependent decrease despite the presence of serum. No osmotic effect of concentration was observed after adjustment of osmolality using mannose. Cell number also tended to decrease with incubation in high concentrations of glucose, suggesting that hyperglycemia itself did not affect proliferation of SMC in accelerated atherosclerosis.[17]

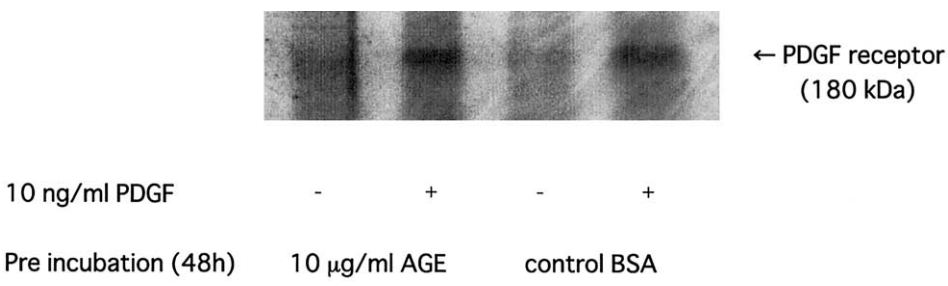

Fig 3. Tyrosine phosphorylation of the PDGF receptor on AGE stimulation. Cultured rat aortic SMC were incubated with 10 μg/mL AGE-BSA or BSA as a control for 48 hours and then stimulated with 10 ng/mL PDGF for 5 minutes at 37°C.

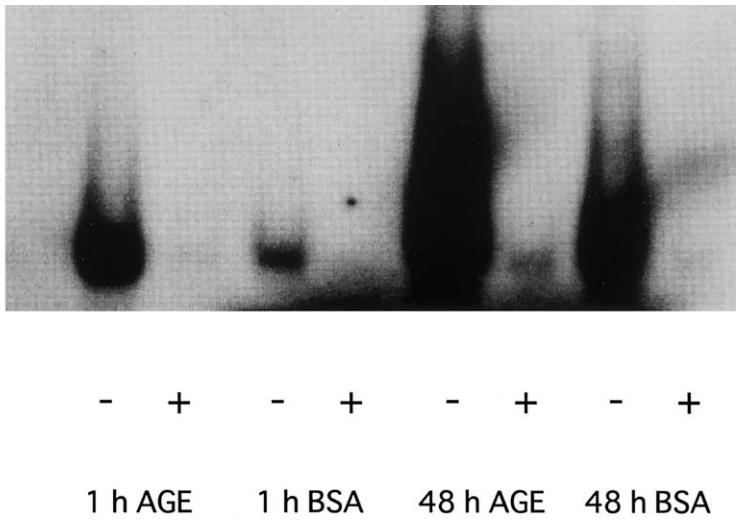

Fig 4. Gel shift mobility assay of NF-κB followings AGE stimulation. Cultured rat aortic SMC were incubated with 10 μg/mL AGE-BSA or BSA control for 1 hour or for 48 hours in DMEM containing 10% FBS.

Incubation at a high glucose concentration was reported to activate protein kinase C (PKC) via de novo synthesis of diacylglycerol.[18] The inhibitory effects of PKC on SMC growth were consistent with previous reports.[19-22] Moreover, oxidative stress was induced by hyperglycemia as a cause of cell damage of high glucose on SMC.[23,24]

We next examined the effect of AGE on proliferation of SMC. In some previous investigations, AGE were implicated in arteriosclerosis.[2,3,25] In our experiment, AGE stimulation increased thymidine uptake by SMC. This effect was observed at concentrations of 5 and 10 μg/mL AGE-BSA. Our result is compatible with the previous observation of a maximal effect of 10 μg/mL AGE on angiogenesis in endothelial cells.[26] On the other hand, high-dose AGE stimulation has been reported to inhibit cell proliferation.[26,27] In our experiment, 150 μg/mL AGE-BSA stimulation decreased thymidine uptake similarly. These data indicate that at relatively low concentrations, AGE may stimulate SMC proliferation. The time course of the stimulatory effect of AGE on SMC proliferation was consistent with the metabolic environment in diabetes, where exposure to hyperglycemia and consequent AGE is chronic.

Stimulatory effects of cell growth by AGE-BSA might be explained by release of cytokines and growth factors by cell types other than SMC.[28,29] We examined whether AGE stimulation attenuates PDGF action in SMC, since PDGF is a major

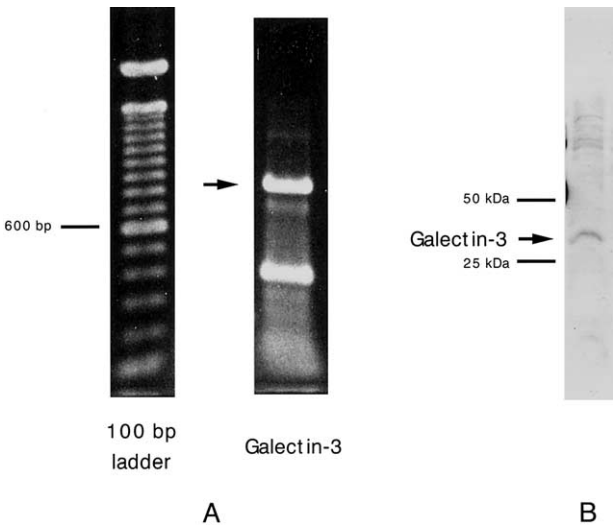

Fig 5. Expression of galectin-3 by cultured rat aortic SMC. (A) Expected sizes of amplified partial rat galectin-3 DNA was 832 base pairs. PCR products were electrophoresed in a 1% agarose gel. (B) Western analysis shows galectin-3 (31 kd) expression on SMC.

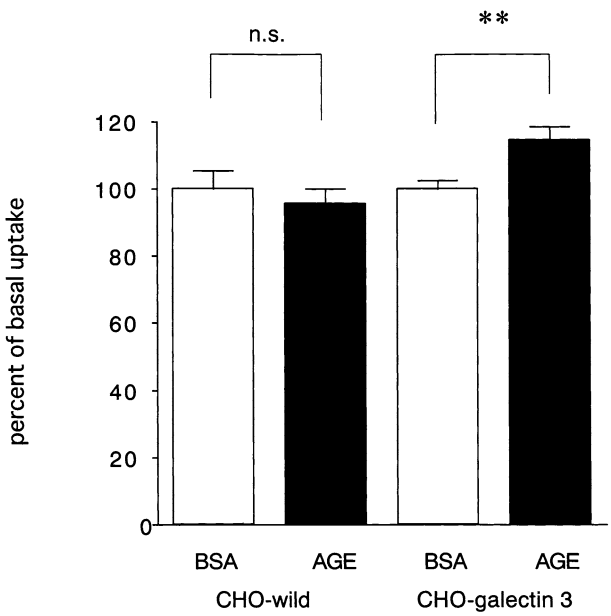

Fig 6. Effect of AGE-BSA on tritium-thymidine uptake by CHO cells. Cells were grown in DMEM containing 5.6 mmol/L glucose and 10% FBS for 5 days and incubated with 10 μg/mL of AGE-BSA for 48 hours in DMEM containing 10% FBS. As controls, the same concentrations of BSA alone were used. Values represent percent of basal uptake. Data are shown as means ± SEM (*$P < .05$; n = 8).

growth factor for proliferation of SMC. We measured autophosphorylation of PDGF receptor because this event is crucial to the first step of signal transduction by growth factors. In our study, tyrosine phosphorylation of the PDGFβ receptor upon stimulation by PDGF was not changed by 48 hours of AGE incubation. Moreover, the time course of the stimulating effect of AGE on SMC proliferation is different from that of PDGF; AGE stimulation has a more prolonged effect (48 hours) than PDGF (12 to 24 hours). These data indicate that the proliferative effects of AGE on SMC are unlikely to result from PDGF receptor activation.

Various possibilities could help to explain SMC proliferation in response to AGE. NF-κB activation has been detected in atherosclerotic lesions.[30,31] NF-κB participates importantly in regulation of gene expression involved in inflammatory and proliferative responses. AGE has been found to activate NF-κB in certain tissues including endothelial cells.[32] AGE-induced NF-κB activation in SMC has been reported.[33,34] In our experiment NF-κB was activated rapidly and time-dependently in SMC by AGE-BSA, which is compatible with the thymidine uptake results in our experiment. AGE-BSA, in our experiment concerning SMC proliferation, had the same effect on NF-κB activation as reported for carboxymethyllysine, an individual AGE.[35] This indicates that activation of NF-κB is an important pathway leading to proliferation of SMC that was stimulated by AGE-BSA.

As another possibility, AGE stimulation has been reported to activate mitogen-activated protein kinase (MAPK) in SMC.[36,37] In those experiments, SMC were stimulated by AGE for only 5 to 10 minutes. In our experiment, AGE stimulation was performed for 48 hours, and MAPK activity did not change significantly for 48 hours (data not shown). These data suggest that short- and long-term mechanisms of response to AGE may differ.

Next, to clarify the mechanism of AGE-BSA–induced cell proliferation, we investigated the role of the AGE receptor in cell proliferation. Various AGE receptors have been identified, such as RAGE,[5] MSR,[6] CD36,[7] galectin-3,[8] and others. AGE receptors expressed by various cells such as macrophages, endothelial cells, mesangial cells, and SMC are thought to influence cell proliferation especially RAGE. Cytokines or growth factors were reportedly released in response to AGE through the AGE receptor, RAGE.[28,29] RAGE has been reported to interact with intracellular signal trunsduction molecules such as NF-κB and/or MAPK.[38] Mechanisms of atherosclerosis involving AGE receptors have been examined in the case of RAGE.[39]

We focused on galectin-3, which has been linked to atherosclerosis.[9,10] We previously reported involvement of galectin-3 in endocytosis of AGE, as well as modified low-density lipoproteins.[12] Expression of galectin-3 was analyzed on masangium cells.[40] In cultured mesangium cells, galectin-3 was not expressed under normal conditions, but it was induced by glucose or AGE stimulation. In our experiment, galectin-3 was natively expressed on SMC under normal (5 mmol/L glucose) conditions. This suggests that expression of galectin-3 in SMC occurred in a manner different from that in mesangium cells. The result that SMC express galectin-3 in the native state suggests that galectin-3 plays a more important role in AGE effects on SMC than on mesangium cells. In our experiments we analyzed the role of galectin-3 using galectin-3–overexpessing CHO cells. AGE stimulation increased thymidine uptake by galectin-3–overexpressing CHO cells. In wild-type CHO cells, which do not express galectin-3,[12] thymidine uptake did not change. These data suggest that SMC proliferation is enhanced by AGE via galectin-3 as an AGE receptor. Pathways involving the AGE–galectin-3 interaction may be among the mechanisms of macroangiopathy in diabetes. Uptake of AGE-BSA has been reported to increase in CHO cells that overexpress both MSR and the insulin receptor,[41] indicating that AGE and hyperinsulinemia in an insulin-resistant state may cooperate as an important influence on SMC proliferation in diabetes-associated atherosclerotic progression.

In conclusion, we demonstrated that AGE but not hyperglycemia itself stimulated SMC proliferation via an AGE receptor. The findings suggest that AGE are important in SMC proliferation in diabetic macroangiopathy. Further investigation should illuminate new targets for preventing diabetic complications.

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