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Advanced Glycation Endproducts Stimulate Mitogen-Activated Protein Kinase and Proliferation in Rabbit Vascular Smooth Muscle Cells

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Advanced glycation end products of bovine serum albumin (AGEs-BSA) exhibited biphasic effects on the proliferation of cultured rabbit vascular smooth muscle cells (VSMCs) in terms of [^{3}H]thymidine incorporation and cell number count; a stimulatory effect was observed at 1-10 μg/ml and an inhibitory effect at more than 20 μg/ml, while it inhibited [^{3}H]thymidine incorporation even at 1-10 μg/ml in cultured bovine vascular endothelial cells (VECs). Transient activation of p42 mitogen-activated protein kinase (MAPK) with a peak at around 5 min and a subsequent sustained phase was induced by AGEs-BSA in VSMCs, but not in VECs. The dependence of MAPK activation on AGEs-BSA dose was correlated with that of VSMCs proliferation.

Diabetes mellitus is an epidemiologically proven risk factor for atherosclerosis (1). Advanced glycation end products (AGEs) of plasma and/or matrix proteins are candidate mediators of diabetic complications such as atherosclerosis and nephropathy (2). Recent epidemiological studies have revealed that the incidence of coronary heart disease is correlated with the plasma concentration of serum albumin AGEs (3). Excess accumulation of AGEs has been immunohistologically detected in atheromatous lesions in diabetic patients (4).

AGEs have been shown to induce diverse biological effects on the various types of cells composing the arterial wall or glomerulus, including cellular activation or dysfunction, growth promotion or cytotoxicity, and regulation of mRNA expression (3). AGEs are reported to stimulate gene expression of platelet-derived growth factor (PDGF) by monocytes (5) and to induce the expression of tissue factor and cytotoxicity in vascular endothelial cells (VECs) (6). AGEs of matrix proteins such as laminin or type IV collagen are known to change the properties and functions of glomerular mesangial cells in culture (7). However, the effects of AGEs on vascular smooth muscle cells (VSMCs) have not been fully elucidated.

A cDNA encoding the cell surface receptor for AGE (RAGE), a member of the immunoglobulin superfamily, has been cloned from a bovine VEC cDNA library (8). RAGE has been immunohistologically detected in VECs, mononuclear phagocytes, glomerular mesangial cells and VSMCs in culture (9). Therefore, some of the actions of AGEs on these cell types are believed to be mediated via this AGE binding to RAGE and the consequent endocytosis of AGEs in these cell types (10). However, some of the actions of AGEs on macrophages were reported to be mediated by scavenger receptors (11). Recently, the other receptor subtype for AGEs responsible for cell chemotaxis was reported (12). Thus, the presence of other receptor subtype for AGEs on VSMCs is still possible. Moreover, the molecular mechanism of AGEs effects in VSMCs have not been demonstrated, although differentiation and mitogenesis of VSMCs are crucial pathogenetic processes in atherosclerosis.

Mitogen-activated protein kinase (MAPK), a family comprising enzymes with molecular masses of 40-58

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Abbreviations used: AGEs, advanced glycation end products; VECs, vascular endothelial cells; VSMCs, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; RAGE, receptor for AGE; DMEM, Dulbecco's modified Eagle's medium, FCS; fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MBP, myelin basic protein; ANOVA, analysis of variance.

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kDa, are activated by phosphorylation of intrinsic tyrosine and threonine residues during differentiation and cell cycle transition triggered by various stimuli, thereby playing a key role in the kinase cascade originating from receptor activation (13). However, the interrelationship between AGE-induced intracellular signaling and MAPK activation has not been investigated.

In this study, we demonstrated for the first time that AGEs-BSA stimulate MAPK activation and proliferation in rabbit VSMCs.

MATERIALS AND METHODS

1. Materials

The reagents used and their commercial sources were as follows: [^{3}H]thymidine (specific activity, 20.1 Ci/mmol), [^{32}P]γ-ATP (specific activity, $>$ 5,000 Ci/mmol), p42/p44 MAPK enzyme assay system from Amersham International plc (Buckinghamshire, England); Toxicolor System from Seikagaku Co. (Tokyo, Japan); Dulbecco's modified Eagle medium (DMEM) from Nissui Pharmaceutical Co. (Tokyo, Japan); RPMI 1640 medium from Bio-Rad (Richmond, CA); fetal calf serum (FCS) from Cell Culture Laboratories (Cleveland, OH); aqueous penicillin and streptomycin from Gibco (Gaithersburg, MD).

2. Preparation of AGEs-BSA

AGEs-BSA were prepared as previously described (14). The formation of AGEs was confirmed by a change in color to brown and positive fluorescence. The contents of pentosidine determined by HPLC assay (15) and endotoxin measured by a Toxicolor system (Seikagaku Co.) in our AGEs-BSA preparation were 10.4 pmol/mg protein and negligible (less than 1 ng/μg protein), respectively. The pentosidine content in the original BSA was not detectable.

3. Cell Culture

Primary cultures of VSMCs and VECs were isolated from the thoracic aorta of female New Zealand White rabbits (4 weeks) by the explant method and from bovine carotid artery by the method of Hirata (16), respectively. The cells were cultured in 75-cm^2 canted-Neck tissue culture flasks with DMEM medium for VSMCs or RPMI-1640 medium for VECs containing 10% heat-inactivated FCS, aqueous penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5 % CO_2 and 95 % air in a humidified atmosphere. Cells used in the experiments were at 5 th to 13 th passage.

4. Assays of Cell Proliferation

[^{3}H]Thymidine incorporation. The cells were seeded at a density of 1×10^4 cells/ml in 6-well plates. Subconfluent cells were incubated with 1 ml of serum-free medium for 24 h to synchronize them at the G_0 stage. Then the cells were incubated with 1 ml medium containing the indicated concentration of AGEs-BSA for 18 h. And then, [^{3}H]-thymidine was added to the final concentration of 1 μCi/ml, and the cells were further incubated for 6 h. [^{3}H]thymidine incorporation into the trichloroacetic acid-insoluble fraction was then determined (17).

Determination of viable cell number. Subconfluent cells (1×10^4 / ml in 6-well plates) were incubated with 1 ml of DMEM (serum-free) for 24 h, and then with 1 ml medium containing the indicated concentration of AGEs-BSA for 48 h. After incubation, the cells were dislodged with trypsin-EDTA, and counted by the trypan blue exclusion test.

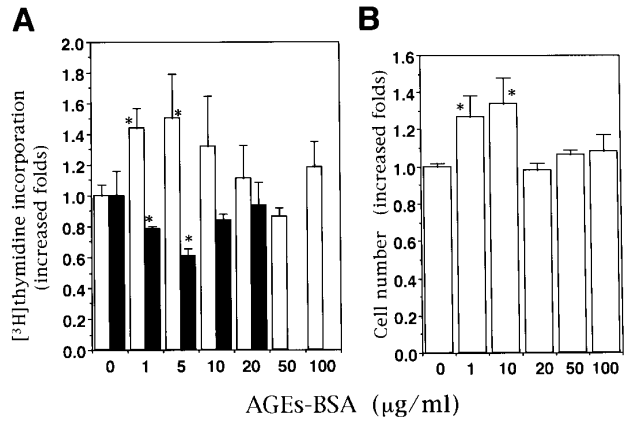

FIG. 1. Effect of AGEs-BSA on cell proliferation in rabbit VSMCs and bovine VECs. (A) Effect of AGEs-BSA on DNA synthesis in rabbit VSMCs (□) and bovine VECs (■). The amount of [^{3}H]thymidine incorporation into DNA was expressed as the ratio relative to the control without AGEs-BSA. The control value was 640 cpm/μg protein in rabbit VSMCs and 9,824 cpm/μg protein in bovine VECs. (B) Effect of AGEs-BSA concentration on proliferation of rabbit VSMCs. The viable cell number per well was expressed as the ratio relative to the control without AGEs-BSA. The cell number in the control was 15,240/well in rabbit VSMCs. Data are expressed as means ± SE. N = 9. *, $P < 0.05$ vs. the control. These results were reproducible in two independent experiments.

5. MAPK Assay

Quiescent cells were washed twice with Tyrode buffer containing 20 mM HEPES (pH 7.4) and 1 mM $CaCl_2$ (HEPES-Tyrode), and then stimulated with AGEs-BSA in HEPES-Tyrode for the indicated time. The cells were lysed in ice-cold lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and centrifuged at 25,000×g for 20 min. The supernatant was used as the source for the assay for MAPK (18).

MAPK assay using the peptide substrate specific for p42/p44 MAPK. The MAPK in the supernatant was measured using the p42/p44 MAPK enzyme assay system according to the manufacturer's instructions (Amersham International plc).

In-gel kinase assay. The supernatant of the cell lysate (150 μl) was immunoprecipitated with anti-MAPK against murine recombinant p42^{mapk} (erk2) with the aid of protein G-Sepharose after heat denaturation of the protein sample. The immunoprecipitates were washed with lysis buffer, and resuspended in the same buffer. In gel kinase assay and analysis using a FUJI BAS 2000 image analysis system were performed as described (18).

6. Statistical Analysis

Statistical analysis of differences was carried out by analysis of variance (ANOVA). A P-value of less than 0.05 was considered to indicate significance. All values are expressed as the means ± SE.

RESULTS

1. Effect of AGEs-BSA on Cell Proliferation in Rabbit VSMCs and Bovine VECs

As shown in Fig. 1 A, AGEs-BSA exhibited a biphasic effect on [^{3}H]thymidine incorporation into DNA in

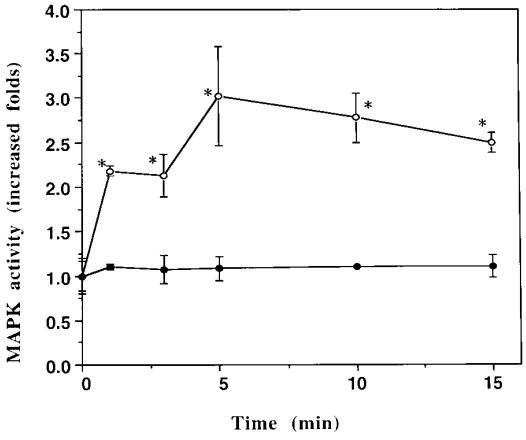

FIG. 2. Time-course of MAPK activation by AGEs-BSA in rabbit VSMCs and bovine VECs. The amount of MAPK activation was expressed as the ratio relative to MAPK activity at 0 min. AGEs-BSA (1 μg/ml) was added at 0 min, as indicated by the arrow. The MAPK activity at 0 min was 10,024 cpm/μg protein in rabbit VSMCs (○) and 1,415 cpm/μg protein bovine VECs (●). Data are expressed as means ± SE. N = 6. *, P < 0.05 vs. the MAPK activity at 0 min. These results were reproducible in two independent experiments.

VSMCs; a significant increase within the range 1 to 5 μg/ml and a dose-dependent decrease at 10-50 μg/ml (Open bars in Fig. 1 A). Non-glycosylated BSA at 1 μg/ml showed no effect on [^{3}H]thymidine incorporation in VSMCs (data not shown). We confirmed the mitogenic effect of AGEs-BSA on VSMCs by cell number counting (Fig. 1 B). The number of viable cells was significantly increased in the presence of AGEs-BSA at 1 to 10 μg/ml, but not significantly changed at 20-100 μg/ml AGEs-BSA. Non-glycosylated BSA at 1 μg/ml showed no effect on the number of VSMCs in comparison with the control wi0thout AGEs-BSA (data not shown). In contrast with rabbit VSMCs, [^{3}H]thymidine incorporation into DNA in VECs was significantly decreased by AGEs-BSA at 1 to 5 μg/ml. In accordance with the results for VSMCs, higher concentrations of AGEs-BSA (10-20 μg/ml) had no significant effect on [^{3}H]thymidine incorporation in VECs (Closed bars in Fig. 1 A). Non-glycosylated BSA at 1-10 μg/ml showed no effect on the number of VECs compared to the control without AGEs-BSA (data not shown).

2. Effect of AGEs-BSA on MAPK Activation in Rabbit VSMCs and Bovine VECs

As shown in Fig. 2, MAPK was activated by 1 μg/ml AGEs-BSA in VSMCs showing a peak at around 5 min of incubation, and then a subsequent sustained phase (Open symbols in Fig. 2), but not activated in VECs at 1-15 min of incubation with 1 μg/ml AGEs-BSA (Closed symbols in Fig. 2). The dose-response effect of AGEs-BSA on MAPK activation revealed a maximal stimulation at 1 μg/ml and a dose-dependent decrease of MAPK activity within the range 10-50 μg/ml (Open bars in Fig. 3 A). In contrast to VSMCs, MAPK in VECs was not activated with 1-50 μg/ml AGEs-BSA (Closed bars in Fig. 3 A).

In order to specify a subtype of MAPK activated by AGEs-BSA, we performed an in-gel kinase assay of MAPK. As shown in Fig. 3 B, a clear band around 42-kDa was phosphorylated in the AGEs-BSA (1 μg/ml)-stimulated cells more than in the control cells, suggesting that a 42-kDa MAPK (MEK) was activated by AGEs-BSA.

DISCUSSION

It is well known that the transformation and mitogenesis of VSMCs in the vascular wall are key steps in the pathogenesis of atherosclerosis (19). Recent studies suggest that AGEs are responsible for the early occurrence and rapid progression of atherosclerosis in diabetic patients (20). However, the effects of AGEs on VSMCs and their intracellular signaling have not been elucidated.

In this study, we demonstrated that AGEs-BSA stimulate the proliferation of VSMCs, as assessed by two criteria; an increase in [^{3}H]thymidine incorporation and that in viable cell number. The stimulatory effect of AGEs on VSMCs proliferation was observed only at the μg/ml level of AGE concentration (Fig. 1). We observed inhibitory effects of AGEs-BSA on the proliferation of VSMCs at more than 50 μg/ml (Fig. 1), even though the inhibition was not statistically significant. We also demonstrated that AGEs at the μg/ml level exhibited significant inhibitory effects on VECs as previously reported (21). Thus, the stimulatory effect of AGEs on cell proliferation is VSMC-specific and AGEs actions may be coupled to multiple signaling systems in VSMCs and VECs.

Activation of MAPK is one of the key events in the pathway from mitogenic stimulation to activation of transcription factors (13,18). Therefore, we tested MAPK activation by AGEs in both VSMCs and VECs. We could detect MAPK activation by AGEs in VSMCs, but not in VECs (Fig. 3 A). The time course of MAPK activation by AGEs-BSA showed a transient peak at around 5 min and a sustained phase at least up to 15 min (Fig. 2), as shown for MAPK activation through tyrosine kinase-receptors or G-protein-coupled receptors or that by direct activation of protein kinase C with a phorbol ester (18). Thus, it is unlikely that this activation of MAPK by AGEs is caused by enhancing gene expression of growth factors such as PDGF (7). The dose-response effect of AGEs on MAPK activation is similar to that on cell proliferation (Figs. 1, 3 A). These results suggest that MAPK activation by AGEs is responsible for the mitogenic response of VSMCs to AGEs. We also confirmed that one of the subtypes of MAPK activated by AGEs in VSMCs is 42-kDa MAPK (MEK), based on the results of an in-gel kinase assay (Fig. 3 B). On the other hand, a cytotoxic effect and no

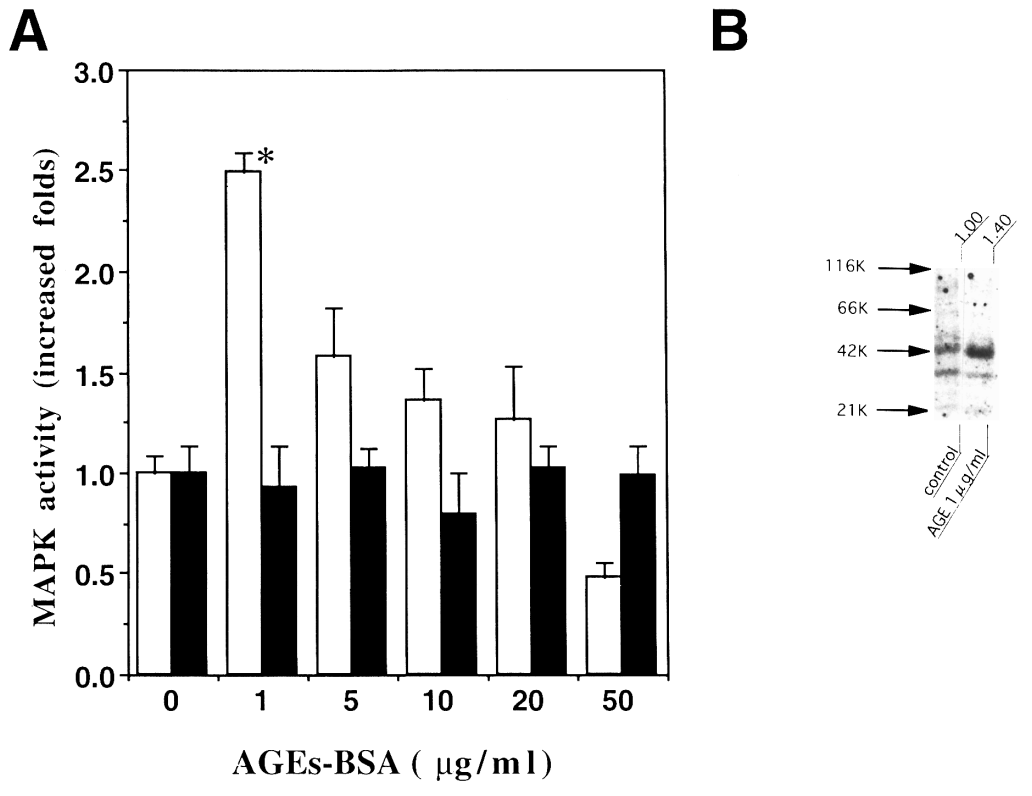

FIG. 3. Effect of AGEs-BSA on MAPK activation in rabbit VSMCs and bovine VECs. (A) Dose-response effect of AGEs-BSA on MAPK activation in rabbit VSMCs (□) and bovine VECs (■). The amount of MAPK activation was expressed as the ratio relative to the control without AGEs-BSA. The control value was 8,824 cpm/μg of protein in rabbit VSMCs and 2,091 cpm/μg protein in bovine VECs. Data are expressed as means ± SE. N = 6. *, $P < 0.05$ vs. the control. These results were reproducible in two independent experiments. (B) In-gel kinase assay of MAPK activation by AGEs-BSA in rabbit VSMCs using anti-rat MAPK monoclonal antibody (erk2). The MAPK was immunoprecipitated with anti-MAPK against murine recombinant $p42^{mapk}$ (erk2). The relative MAPK activity shown at the top was expressed as the ratio relative to the control without AGEs-BSA.

MAPK activation were observed in VECs at the AGE concentrations that stimulated the proliferation and MAPK activation in VSMCs.

At this moment, it is still unclear whether the differences in cellular responses to AGEs between VSMCs and VECs are due to the difference in receptor subtypes activated by AGEs or those in the intracellular machinery triggered by AGEs. Nevertheless, our results provide a new insight into the pathophysiological mechanism of atherosclerosis in patients with diabetes.

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