

Advanced glycation end products induce actin rearrangement and subsequent hyperpermeability of endothelial cells

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This study aimed to determine the effects of advanced glycation end products (AGEs) on endothelial cytoskeleton morphology and permeability, and to detect the underlying signaling mechanisms involved in these responses. Cultured endothelial cells (ECs) were exposed to AGE-modified human serum albumin (AGE-HSA), and EC cytoskeletal changes were evaluated by observing fluorescence of F-actin following ligation with labeled antibodies. Endothelial permeability was detected by measuring the flux of TRITC-albumin across the EC monolayers. To explore the signaling pathways behind AGE-induced EC alteration, ECs were treated with either soluble anti-AGE receptor (RAGE) IgG, or the MAPK inhibitors PD98059 and SB203580 before AGE-HSA administration. To further elucidate possible involvement of the ERK and p38 pathways in AGE-induced EC changes, adenovirus-carried recombinant constitutive dominant-negative forms of upstream ERK and p38 kinases, namely MEK1(A) and MKK6b(A), were pre-infected into ECs 24 h prior to AGE-HSA exposure. AGE-HSA induced actin cytoskeleton rearrangement, as well as EC hyperpermeability, in a dose and time-dependent manner. The effects were attenuated in cells pretreated with anti-RAGE IgG, PD98059 or SB203580, respectively. EC pre-infection with MEK1(A) and MKK6b(A) also alleviated the effect of AGEs. Furthermore, adenovirus-mediated administration of activated forms of either MEK1 or MKK6b alone induced rearrangement of F-actin and hyperpermeability. The results indicate that ERK and p38 MAPK play important roles in the mediation of AGE-induced EC barrier dysfunction associated with morphological changes of the F-actin.

Key words: Advanced glycation end products (AGEs); endothelial cells; actin; cytoskeleton; MAPK.

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Advanced glycation end products (AGEs) form following irreversible non-enzymatic reactions between glucose carbonyls and protein amino groups, particularly on long-lived proteins such as collagens (1). Accumulation of AGEs *in vivo* has been found to increase with age and to occur at an accelerated rate in subjects with par-

ticular pathological conditions, such as diabetes, where levels of AGEs are correlated with the severity of complications elicited (2–4). The progressive accumulation of AGEs in diabetes patients suggests that endogenous clearance mechanisms fail to function effectively at sites of AGE deposition. Deposited AGEs may have the capacity to alter cellular properties by a number of mechanisms. Direct effects of AGEs in the extracellular space include formation of

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crosslinks that may also trap neighboring unrelated macromolecules. Furthermore, AGEs seem to induce signals that exert complex effects on cellular functions via a complicated transduction system (2–4).

There is substantial evidence for a link between AGEs and the initiation and development of multiple-organ damage, especially microvascular and macrovascular complications in diabetes, Alzheimer's disease, arteriosclerosis, tumors and nephropathy (5–7). Recent studies (8) have demonstrated that AGEs significantly increase vascular permeability, which is a characteristic feature of early vasculopathies of those diseases.

It is well known that endothelial cells (ECs) play a key role in the modulation of vascular permeability. ECs form a semipermeable dynamic barrier that regulates the exchange of fluids and solutes between the vascular space and underlying tissues. Disruption of the endothelial monolayer, or disassociation of intercellular junctions, leads to barrier dysfunction and edema. The barrier function requires the tight adherence of ECs to maintain the integrity and continuity of the monolayer, which are balanced by contractile forces and adhesive cell-cell and cell-matrix tethering forces (9). Generation of contractile forces within the endothelial cells can result in opening of intercellular gaps and, thereby, changes in endothelial barrier function. One of the most important regulating mechanisms of the contractile machinery of endothelial cells is rearrangement of the cytoskeleton, which is composed of myosin, actin and other components. Myosin light chain kinase (MLCK) is involved in the adjustment of the endothelial barrier permeability via its activation by phosphorylation that induces actin-myosin-based contraction (10–12). When the interaction between myosin and actin is increased, the contractile forces predominate and enhanced paracellular permeability follows.

There are two forms of cellular actin, globular actin (G-actin) and filamentous actin (F-actin). Under physiological conditions, G-actin readily polymerizes to form F-actin with a concomitant hydrolysis of ATP. By polymerizing or depolymerizing, G-actin and F-actin interact to maintain a dynamic balance. F-actin rearrangement is characterized by polymerization of F-actin to form central stress fibers with ensuing

disruption of the peripheral actin rim (PAR) (13, 14). It is thus likely that endothelial cell retraction would be accompanied by alterations of endothelial F-actin organization. Despite some proof that AGE deposits may increase vascular permeability (8, 15), little is known about AGE-induced changes of F-actin assembly that would link cytoskeleton alterations to hyperpermeability.

It is believed that AGEs exert their effects by binding to a specific receptor (RAGE) on the surface of target cells (16, 17). AGEs themselves upregulate the RAGE expression in microvascular ECs (18). The engagement of RAGE by AGEs induces an activation of ECs, including expression of the vascular cell adhesion molecule-1 (VCAM-1) (19), generation of reactive oxygen species (20), and increased permeability (15) in cultured human ECs. It is also proposed that the binding of AGEs to RAGE triggers cascades of intracellular signaling events, including the activation of Ras (21) and MAP kinases (22), while its detailed signaling mechanisms have not been fully explored. In this study, we found AGE-induced EC actin rearrangements with subsequent alteration of EC permeability, and that the ERK and p38 MAP kinase pathways are involved.

MATERIALS AND METHODS

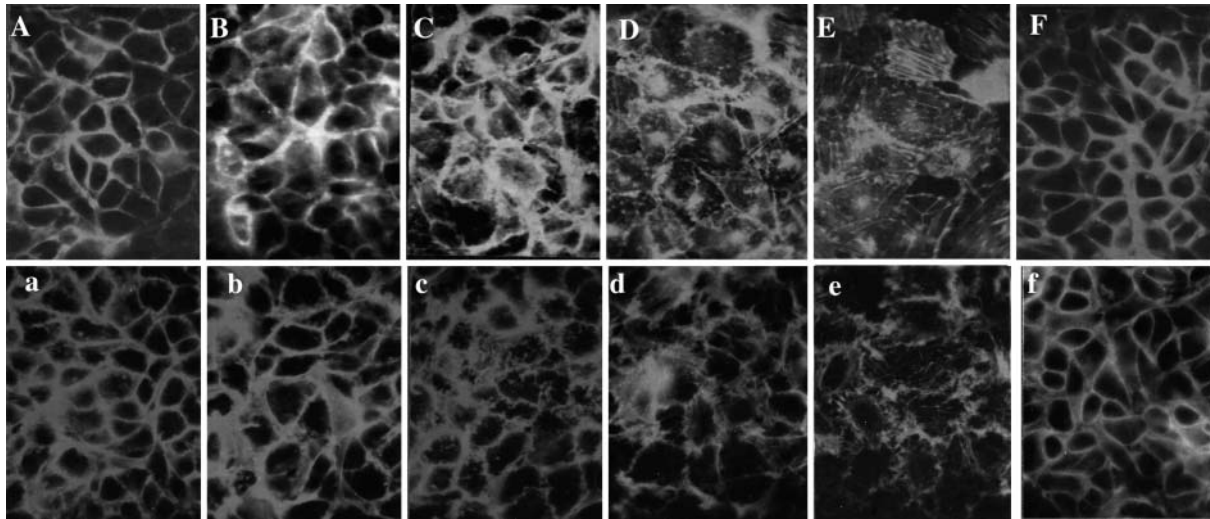
Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. The MAPK inhibitors PD98059 (a MEK1 specific inhibitor) and SB203580 (a p38 specific inhibitor) were acquired from Calbiochem (San Diego, CA, USA). Rhodamine-phalloidin was from Molecular Probe (Carlsbad, CA, USA). DMEM and trypsin were from Gibco BRL (Carlsbad, CA, USA), while bovine serum was from Hangzhou Sijiqing BioProduct Co. Ltd. (China). Monoclonal antibodies against different epitopes of the AGE extracellular domain receptor (Anti-RAGE IgG) were prepared and characterized according to Zhu (23). Adenovirus with recombinant constitutive dominant-negative forms of upstream ERK and p38 kinases MEK1(A) and MKK6b(A) were kindly provided by Dr. Han Jiahuai at the Scripps Research Institute, La Jolla, USA.

Preparation of AGE-HSA

AGE-HSA was prepared according the protocol of Hou et al. (24, 25). Briefly, human serum albumin (150 mmol/L, pH 7.4) was incubated in PBS with D-

Fig. 1. AGE-HAS induces actin reorganization in endothelial cells in a dose- and time-dependent manner.



Endothelial cells were grown to confluence on gelatin-coated glass-bottom microwell Petri dishes. Cells were then exposed to 0 (A), 12.5 (B), 25 (C), 50 (D), or 100 (E) $\mu\text{g/ml}$ AGE-HSA for 8 h, while cells in lane F were exposed to 100 $\mu\text{g/ml}$ HSA (no AGE). ECs were also exposed to 50 $\mu\text{g/ml}$ AGE-HSA for 2 (a), 4 (b), 8 (c), 12 (d), and 24 h (e), respectively. Cells in lane f were exposed to 50 $\mu\text{g/ml}$ HSA only for 24 h. F-actin was stained with rhodamine-phalloidin.

glucose (250 mmol/L) at 37°C for 6 weeks. Control albumin was incubated without glucose. At the end of the incubation period, both solutions were extensively dialyzed against PBS and purified. Endotoxin content was detected by a *limulus amoebocyte* lysate assay (Sigma, St. Louis, MO, USA) and was found to be less than 0.05 U/mL (5 pg/mL) in both solutions. AGE-specific fluorescence was determined by using ratio spectrofluorometry. AGE-HSA contained 74.80 U/mg protein AGE, while native albumin contained 0.9 U/mg protein AGE.

Cell culture

A human umbilical vein endothelial cell line (ECV304) was purchased from the Cell Institute of the Chinese Scientific Academy in Shanghai, cultured in complete DMEM with 10% heat-inactivated newborn bovine serum, 100 u/ml penicillin, and 125 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified 5% CO₂ incubator.

Fluorescent staining of F-actin

ECs were plated in gelatin-coated glass-bottom microwells (MatTek, MA, USA) at a density of 75,000 to 100,000 cells/ml and cultured to confluence. Cells were then rendered quiescent in serum-free medium for 12 h, followed by appropriate treatments. They were fixed and permeated for 15 min at room temperature in PBS with 3.7% formaldehyde and 0.5% Triton X-100. They were then incubated with rhodamine-phalloidin (2 unit/ml) for 40 min at room temperature to stain F-actin. Finally, they were washed three times with chilled PBS, mounted to

allow fluorescence microscopy, and observed with appropriate rhodamine-phalloidin filters. Images were recorded using Kodak 400 films. Since there is no good way of objectively analyzing the alterations in F-actin distribution, a large number of micrographs were taken and representative ones were used for printing.

Endothelial monolayer permeability assay

Endothelial monolayer permeability was measured as described by Tinsley (26). ECs were grown to confluence on 1% gelatin-coated transwell-clear polyester membranes (Corning Costar, Acton, MA, USA). Cells were starved in serum-free medium for 12 h and then exposed to different reagents as indicated, or adenovirus infected with recombinant kinases before exposure to AGEs. Tracer protein TRITC-albumin (1 mg/ml) was then added to the upper chambers for 45 min. Samples were collected from both the upper (luminal) and lower (abluminal) chambers for fluorometry analysis. Albumin concentrations were detected using a HTS 7000 microplate reader (Perkin-Elmer, Yokohama, Japan) and a standard curve. The permeability coefficient for albumin (P_a) was calculated as: $P_a = [A]/t \cdot 1/A \cdot V/[L]$, where [A] is the abluminal albumin concentration, t is the time in seconds, A is the area of the membrane in cm², V is the volume of the abluminal chamber, and [L] is the luminal albumin concentration.

Statistical analysis

For each experiment, P_a was measured more than three times and values were normalized as percentage

of controls (mean \pm SD). One-way ANOVA was used to evaluate the significance of inter-group differences. $p < 0.05$ was considered statistically significant.

RESULTS

AGE-HSA induces changes in the EC actin cytoskeleton

Continuous F-actin was mainly distributed in the cortical area of ECs under normal conditions, forming a typical peripheral actin rim (PAR) (13, 14). Few stress fibers were seen (Fig. 1A). Exposure of ECs to AGE-HSA caused a shift in F-actin distribution from a web-like structure to polymerized stress fibers in a concentration- (Fig. 1B–E) and time-dependent manner (Fig. 1a–e). The administration of 50–100 μ g/ml HSA alone for 24 h had no effects on the distribution of EC F-actin (Fig. 1F & 1f).

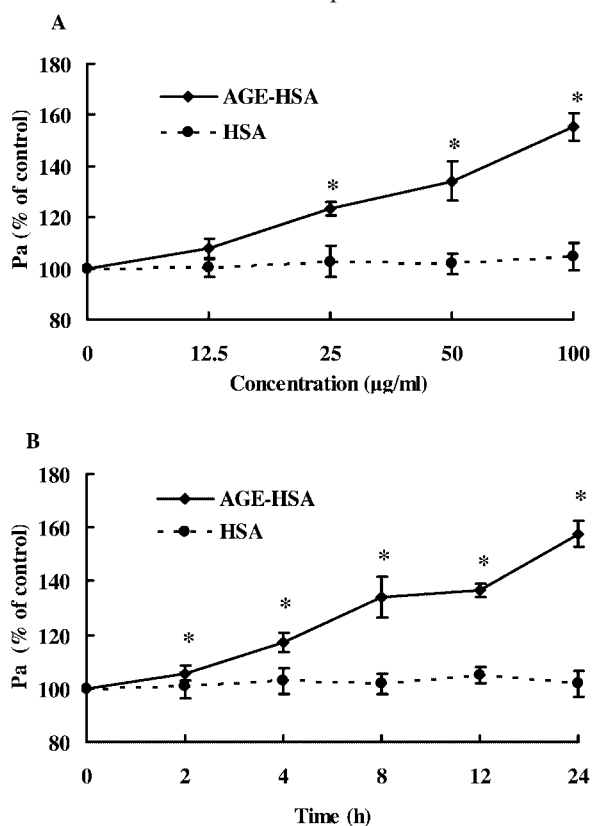
AGE-HSA causes hyperpermeability in EC monolayers

Exposure of ECs to AGE-HSA induced a significant elevation of the permeability coefficient for TRITC-albumin (P_a) in a dose- and time-dependent manner. Exposure to 12.5, 25, 50 or 100 μ g/ml of AGE-HSA increased P_a to $107.68 \pm 3.76\%$, $123.24 \pm 2.55\%$, $134.06 \pm 7.70\%$ and $155.48 \pm 5.30\%$ of control cells, respectively (Fig. 2A). The permeability response occurred within 2 h after a 50 μ g/ml AGE-HSA exposure, and P_a increased continually for the rest of the observation period. The P_a value at 24 h was $157.82 \pm 4.81\%$ of the control value (Fig. 2B). Incubation with HSA alone had no effect on EC permeability.

Signaling pathways are involved in AGE-HSA-induced EC alterations

To elucidate signal pathways that might be involved in AGE-HSA-induced EC alterations, ECs were pretreated with anti-RAGE IgG and the MAPK inhibitors PD98059 and SB203580, respectively, before exposure to AGE-HSA. There was less stress fiber formation in pretreated ECs and the peripheral actin rim (PAR) was well preserved as compared to ECs exposed to AGE only. Anti-RAGE IgG, PD98059 and SB203580 also abolished the AGE-HSA-induced redistribution of F-actin (Fig. 3), while the negative control reagent for SB203580,

Fig. 2. AGEs increase EC monolayer permeability in a concentration- and time-dependent manner.

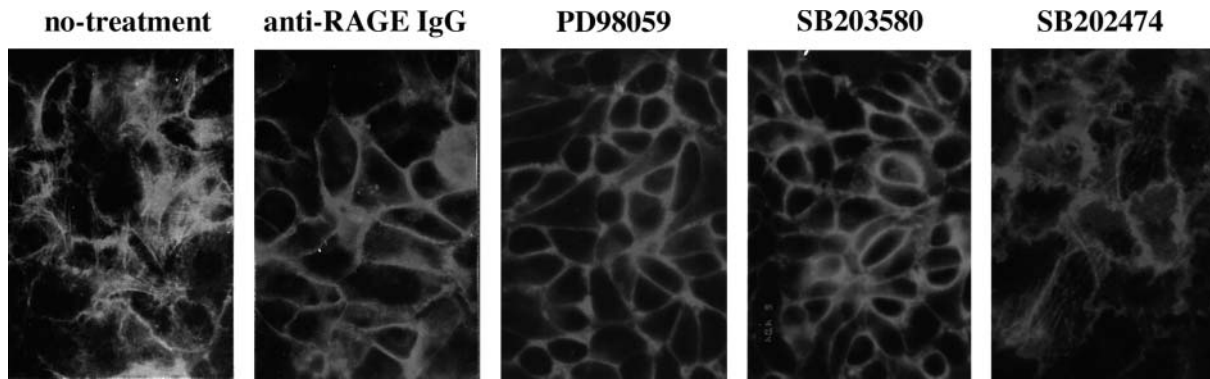


Confluent EC monolayers, grown on transwell inserts, were treated with AGE-HSA. *Panel A*: ECV304 exposed to 0, 12.5, 25, 50 or 100 μ g/ml AGE-HSA or HSA only for 8 h. *Panel B*: P_a was assessed 0, 2, 4, 8, 12 and 24 h after the addition of 50 μ g/ml AGE-HSA or HSA only. TRITC-albumin was then added to the luminal chamber and permeability of albumin (P_a) was calculated after 45 min. Data are shown as percentage of control (mean \pm SD, $n=3$). * indicates significant difference compared with the basal value in the absence of AGE-HSA.

SB202474, a similar compound with no inhibitory effect on p38, failed to attenuate formation of stress fiber and disruption of F-actin, suggesting a specific involvement of p38 activation during AGE-induced actin reorganization. Anti-RAGE IgG and SB203580 themselves had no effect on ECs actin morphology (data not shown).

Anti-RAGE IgG and the MAPK inhibitors prevented AGE-HSA-induced hyperpermeability. Pretreatment of EC monolayers with soluble anti-RAGE IgG for 1 h attenuated AGE-HSA-induced elevations in P_a in a con-

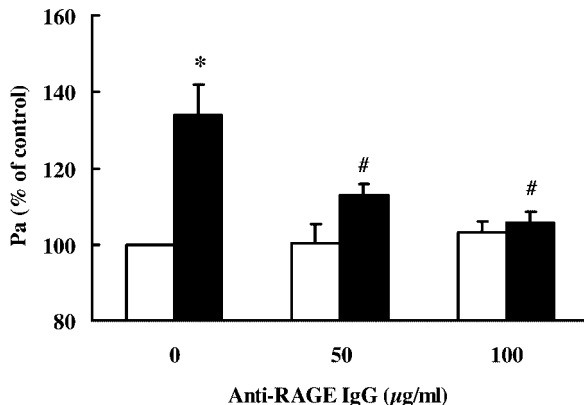
Fig. 3. Inhibition of AGE-HSA-induced morphological changes by a RAGE antibody and ERK and p38 MAPK inhibitors in endothelial cells.



ECs were pretreated as indicated: 100 $\mu\text{g/ml}$ anti-RAGE IgG for 1 h, 100 $\mu\text{mol/L}$ PD98059 or 25 $\mu\text{mol/L}$ SB203580 for 30 min. A mimic compound (which has no inhibition function), SB202474, was applied as a negative control. Cells were then exposed to 50 $\mu\text{g/ml}$ AGE-HSA for 8 h. F-actin was stained with rhodamine-phalloidin.

centration-dependent manner. Anti-RAGE IgG (50 and 100 $\mu\text{g/ml}$) reduced P_a from $134.06 \pm 7.70\%$ to $113.34 \pm 5.04\%$ and $105.74 \pm 3.06\%$ of control, respectively, in the presence of 50 $\mu\text{g/ml}$ AGE-HSA (Fig. 4), while it did not affect baseline permeability in the absence of AGE-HSA, suggesting that binding of AGE to RAGE plays

Fig. 4. Blocking RAGE with anti-RAGE IgG attenuates AGE-HSA-induced hyperpermeability in a dose-related manner.



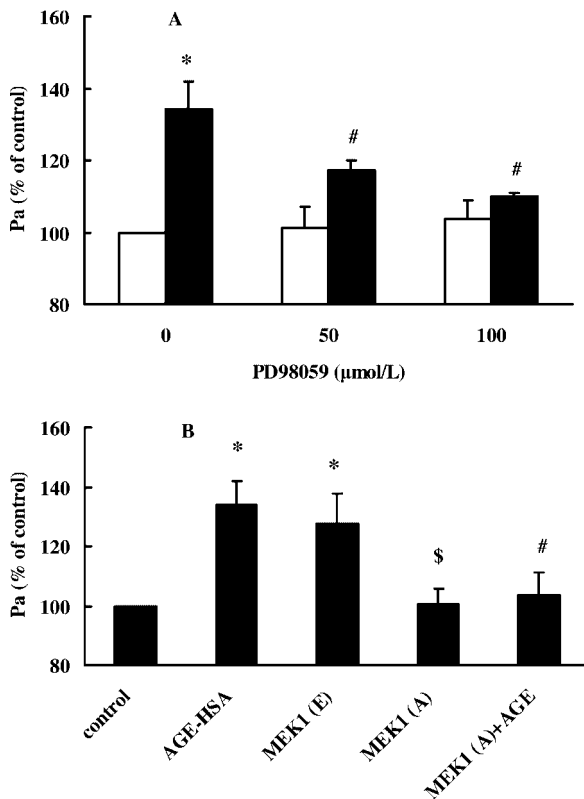
Confluent ECs were pretreated for 1 h with anti-RAGE IgG at indicated concentrations, followed by treatment with either diluents (open bars) or AGE-HSA (50 $\mu\text{g/ml}$; filled bars) for another 8 h. P_a values are shown as percentage of control (mean \pm SD, $n = 3$). * indicates significant difference compared with the basal value in the absence of AGE-HSA. # indicates significant difference compared with ECs treated with AGE-HSA only.

an important role in the mediation of AGE-induced increase of endothelial permeability.

Pretreatment of ECs with a specific inhibitor of the ERK pathway, PD98059 (50 and 100 $\mu\text{mol/L}$), or a specific inhibitor of p38 MAPK, SB203580 (10 and 25 $\mu\text{mol/L}$), both abolished AGE-HSA-induced hyperpermeability, as P_a was reduced from $134.06 \pm 7.70\%$ to $117.09 \pm 2.74\%$ and $110.02 \pm 0.92\%$ or $120.34 \pm 9.79\%$ and $107.25 \pm 7.58\%$, respectively (Figs. 5A & 6A). The extent of P_a reduction was related to the applied concentration of PD98059 or SB203580, respectively, suggesting a specific abolishing effect of those reagents on AGE-HSA signaling pathways.

To further elucidate the involvement of the ERK and p38 pathways in AGE-induced EC changes, recombinant constitutive dominant-negative forms of upstream ERK and p38, namely MEK1(A) and MKK6b(A) kinases, were transfected to ECs by an adenoviral carrier, 24 h before AGE-HSA treatment, aiming at inhibition of ERK and p38 phosphorylation and activation. Monolayer permeability was then measured. MEK1(A) and MKK6b(A) induced potent inhibitory effects on the hyperpermeability response induced by AGE-HSA exposure (Figs. 5B & 6B). The hyperpermeability response was also attenuated by inactivation of the p38 pathway through infection with the adenovirus carrying recombinant dominant-negative form of p38 α , [p38 α (A)], while the adeno-

Fig. 5. Inhibition of the ERK MAPK pathway abolishes AGE-induced permeability increase.

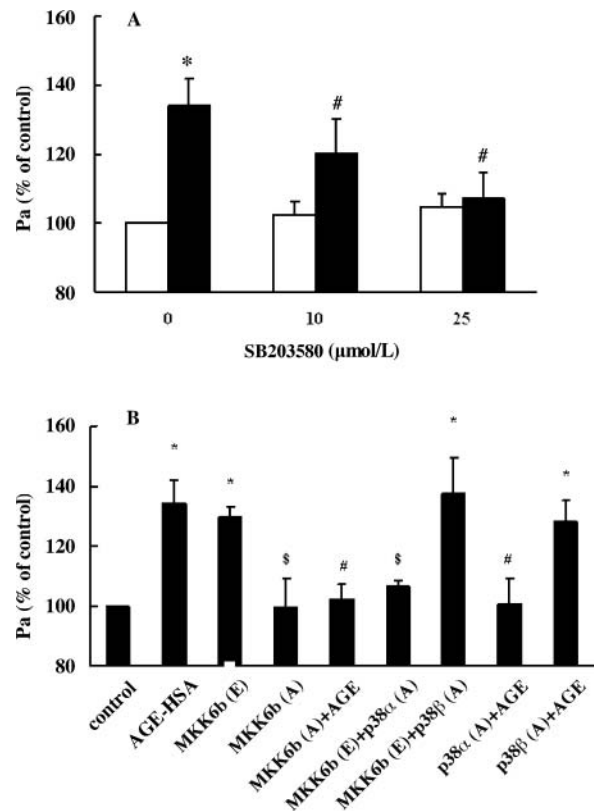


Panel A: Confluent ECs were pre-incubated with diluent (control) or PD98059 at indicated concentrations for 30 min, followed by treatment with either diluent (open bars) or AGE-HSA (50 μg/ml; filled bars) for another 8 h. **Panel B:** Confluent ECs were pre-incubated with diluent, or pre-infected with MEK1(E) or MEK1(A) for 24 h, followed by treatment with either diluent or AGE-HSA (50 μg/ml) for 8 h. Data are given as percentage of control *Pa* (no virus, no AGE, mean±SD, n=3). * indicates significant difference compared with the basal value in the absence of AGE-HSA. # indicates significant difference compared with ECs treated with AGE-HSA only. \$ indicates significant difference compared with ECs treated with MEK1 (E) only.

virus carrying recombinant dominant-negative form of p38β, [p38β(A)] had no effects on AGE-induced high permeability response in EC monolayers.

Furthermore, recombinant constitutive active forms of MEK1 or MKK6b [MEK1 (E) or MKK6b (E)] were adenovirus-infected to ECs in order to mimic the activation of ERK and p38 pathways induced by AGE-HSA. The results showed that *Pa* increased to 127.63±10.19% and 129.60±3.56% of control, respec-

Fig. 6. Inhibition of the p38 MAPK pathway attenuates AGE-induced permeability increase.



Panel A: Confluent ECs were pre-incubated with diluent (control) or SB203580 at indicated concentrations for 30 min, followed by treatment with either diluent (open bars) or AGE-HSA (50 μg/ml; filled bars) for 8 h. **Panel B:** Confluent ECV304 were pre-incubated with diluent; or pre-infected with MKK6b (E), MKK6b (A), p38α (A) or p38β (A) for 24 h, followed by treatment with either diluent or AGE-HSA (50 μg/ml) for 8 h; or co-infected with MKK6b (E) plus p38α (A) or p38β (A) for 24 h, followed by treatment with diluent. Data are shown as percentage of control *Pa* (no virus, no AGE, mean±SD, n=3). * indicates significant difference compared with the basal value in the absence of AGE-HSA. # indicates significant difference compared with ECs treated with AGE or. \$ indicates significant difference compared with ECs treated with MKK6b (E) only.

tively (Figs. 5B & 6B), while the effect elicited by MKK6b (E) was eliminated by co-infection with p38α (A). As expected, p38β(A) failed to exert any abolishing effect on MKK6b (E)-induced hyperpermeability (Fig. 6B). These results indicate that ERK and p38α are indeed involved in the AGE-induced endothelial monolayer permeability response.

DISCUSSION

The results of the present study clearly indicate that AGE-HSA increases endothelial monolayer permeability in a concentration- and time-dependent manner, and that the hyperpermeability is paralleled by morphological changes of the actin cytoskeleton.

It is well known that phosphorylation of MLC by MLCK is essential and sufficient for initiation of endothelial cell retraction (10). Since actin, myosin and the functional correlate of the activated actomyosin compose the contractile system in ECs (11, 12), we assessed EC retraction in terms of changes in cytoskeletal F-actin distribution.

Our results strongly support the idea that actin rearrangement is a major cause of barrier dysfunction in endothelial cells. The actin cytoskeleton of non-muscle cells responds to extracellular stimuli through spatially and temporally regulated series of polymerization and depolymerization reactions and by enhanced actomyosin interaction, which all contribute to the generation of contractile forces. Actin polymerization plays a key role in the determination of cell shape, which in turn has a great impact on EC permeability. Here, we found that F-actin underwent morphological alterations after AGEs stimulation, indicating that polymerization and depolymerization transformations were activated. Furthermore, F-actin communicates with intercellular junctions, such as adherence junctions and tight junctions, which both contribute to adhesive forces and the maintenance of endothelial cell integrity (27–29). Disassembly of PAR may have a disorganization effect on intercellular junctions, in turn resulting in the observed EC retraction with subsequent gap formation and enhanced paracellular flux of water and albumin. Our observations suggest that the VE-cadherin complex might be destroyed following exposure to AGEs (30), which may result from eruption of actin, resulting in increased contractile forces and a decrease of tethering forces.

The increased protein leakage through EC monolayers when exposed to AGEs coincides with the results of previous studies performed in microvessels (16). However, little is known about the cellular mechanisms for such EC barrier dysfunctions.

It is known that AGEs mainly exert their cellular effects via a direct interaction with cells through a specific binding system. There are several AGE receptors, which have been identified in a wide range of cells and tissues. The best characterized AGE receptor is RAGE, which is a multiligand member of the immunoglobulin G family (31). Engagement of AGEs to RAGE does not accelerate clearance or degradation but rather begins a sustained period of cellular activation mediated by receptor-dependent signaling. Moreover, AGE ligation also enhances receptor expression and initiates a positive feedback loop, whereby receptor occupancy triggers increased RAGE expression and perpetuates another wave of cellular activation (32). This last point is crucial, because it explains how upregulation of this receptor contributes to an ascending spiral of RAGE-dependent cellular perturbations. Vascular endothelium is known to bind and internalize AGEs through RAGE. Since an AGE-RAGE interaction is thought to play a central role in mediating signal transduction, we employed anti-RAGE-IgG that directly blocks the binding of AGEs to RAGE to test the role of the latter in vascular dysfunction. As expected, pre-exposure to anti-RAGE-IgG significantly reduced the hyperpermeability otherwise induced by AGEs in a concentration-dependent manner.

MAPK is a major signaling system that transduces a variety of extracellular signals through a cascade of intracellular protein phosphorylation processes and plays an important role in the regulation of cell growth, differentiation, apoptosis, and cellular response to environmental stress. In mammals, four major subgroups of the MAPK superfamily members have been identified: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK 5 (33, 34).

It is reported that cellular oxidant stress consequent to AGE-RAGE interaction would trigger the p21^{ras}/ERK MAP kinase pathway. The molecular mechanism may be a consequence of triggering p21^{ras} exchange activity by reactive oxygen modification of Cys¹¹⁸ on p21^{ras}, rapidly followed by ERK MAP kinase pathway activation (18). Evidence has been found that ERK1/2 activation reaches its peak within 15 to 20 min in cells exposed to AGEs

(35). In addition, activated RAGE binds directly to ERK by a D-domain-like docking site, followed by activation of ERK (36). To test the role of ERK MAPK in the pathology process stimulated by AGEs, we used the specific MEK (activator of ERK) inhibitor PD98059. The result was a significant increase in permeability as well as an augmented formation of actin stress fibers in response to AGEs that was attenuated by PD98059 in a dose-dependent manner. Additionally, transfection of ECs with MEK1(E) induced actin stress fiber formation associated with hyperpermeability, whereas infection by MEK1(A) abolished these responses. These findings potently suggest that ERK MAPK is involved in intercellular signal pathways evoked by AGEs. The further downstream effectors, leading to hyperpermeability in AGEs-stimulated endothelium, are less well understood, but as shown in other experiments, initiation of the ERK pathway-mediated activation of MLC kinase (37), which phosphorylates MLC to produce intracellular contractile force, causes stress fiber formation and hyperpermeability by increasing the ATPase activity of myosin (38, 39).

Our previous studies on EC cultures incubated with AGEs demonstrated that p38 phosphorylation activity is clearly enhanced within 10 min, reaching a peak after 30 min (40). It is well known that p38 MAPK signaling is most sensitive to inflammatory responses. The signal relay along the p38 pathway involves a kinase cascade, which generally consists of the upstream stimulators MAPK kinase 6 (MKK6) and 3 (MKK3), the p38 kinases (α , β , γ and δ), and their downstream effectors. MKK6b is an isoform of MKK6 that is recognized as a primary upstream kinase of p38 α , also called p38, which has more than a 60% sequence identity in comparison to three other p38 isoforms (p38 β , γ and δ). p38 α and p38 β are ubiquitously expressed in endothelial cells and specifically inhibited by SB203580. One of the p38 downstream protein kinases is the MAPK-activated protein kinase 2 (MK2) that phosphorylates heat shock protein 27 (Hsp27) (41). The latter is an actin-binding protein, whose actin polymerization-inhibiting activity dramatically decreases after phosphorylation in association to stress fiber development. Additionally, several groups have previously demonstrated that p38 MAPK activation results in phosphorylation of

an actin and myosin-binding protein, caldesmon, which interferes with actin stress fiber formation, actomyosin interaction and cell contraction (42).

We have here shown that both SB203580 and the dominant-negative p38 α vector, while not that of p38 β , significantly attenuated AGE-induced hyperpermeability and actin rearrangement, suggesting that a p38 MAPK-dependent regulation of actin dynamics is responsible for AGE-induced EC hyperpermeability and that p38 α is the involved subtype. Furthermore, infection with the dominant active form of MKK6b caused hyperpermeability and reorganization of the actin cytoskeleton in endothelial cells in a similar way to that of AGEs.

In summary, the present study demonstrated that the AGE-induced increase in EC permeability is associated with morphological changes of the F-actin cytoskeleton, which plays an important role in regulating the endothelial barrier. This study also provides direct evidence for roles of ERK and p38 MAPK in mediation of AGE-induced EC barrier dysfunction. Ligation of AGE to its receptor RAGE might trigger AGE-elicited hyperpermeability responses.

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REFERENCES

1. Brownlee M, Vlassara H. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* 1995;46:223–34.
2. Forbes JM, Yee LTL, Thallas V, Lassila M, Candido R, Jandeleit-Dahm KA, et al. Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 2004; 53:1813–23.
3. Peppas J, Uribarri J, Vlassara H. Glucose, advanced glycation end products, and diabetes complications: what is new and what works. *Clinical Diabetes* 2003;21:186–7.
4. Thomalley PJ. Cell activation by glycated proteins. AGE receptor, receptor recognition factors

- and functional classification of AGEs. *Cell Mol Biol* 1998;44:1013–23.
5. Vlassara H, Bucala R, Striker L. Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* 1994;70:138–51.
 6. Li YM, Steffes M, Donnelly T, Liu C, Fuh H, Basgen J, et al. Prevention of cardiovascular and renal pathology of aging by the advanced glycation inhibitor aminoguanidine. *Proc Natl Acad Sci USA* 1996;93:3902–7.
 7. Stitt AW, He C, Friedman S, Scher L, Rossi P, Ong L, et al. Elevated AGE-modified ApoB in sera of euglycemic, normolipidemic patients with atherosclerosis: relationship to tissue AGEs. *Mol Med* 1997;3:617–27.
 8. Leto G, Pricci F, Amadio L, Iacobini C, Cordone S, Diaz-Horta O, et al. Increased retinal endothelial cell monolayer permeability induced by the diabetic milieu: role of advanced non-enzymatic glycation and polyol pathway activation. *Diabetes Metab Res* 2001;17:448–58.
 9. Dudek SM, Garcia JN. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487–500.
 10. Wysolmerski RB, Lagunoff D. Involvement of myosin light chain kinase in endothelial cell retraction. *Proc Natl Acad Sci USA* 1990;87:16–20.
 11. Goeckeler ZM, Wysolmerski RB. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *J Cell Biol* 1995;130:613–27.
 12. Sheldon R, Moy A, Lindsley K, Shasby S, Shasby DM. Role of myosin light-chain phosphorylation in endothelial cell retraction. *Am J Physiol* 1993;265:L606–12.
 13. Ermert L, Bruckner H, Walrath D. Role of endothelial cytoskeleton in high-permeability edema due to botulinum C2 toxin in perfused rabbit lungs. *Am J Physiol* 1995;268:L753–61.
 14. Thurston G, Baldwin AL. Changes in endothelial actin cytoskeleton in venules with time after histamine treatment. *Am J Physiol* 1995;269:H1528–37.
 15. Otero K, Martinez F, Beltran A, Gonzalez D, Herrera B, Quintero G, et al. Albumin-derived advanced glycation end-products trigger the disruption of the vascular endothelial cadherin complex in cultured human and murine endothelial cells. *Biochem J* 2001;359:567–74.
 16. Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, et al. Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. *J Clin Invest* 1996;97:238–43.
 17. Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, et al. Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci USA* 1994;91:8807–11.
 18. Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y, Yamamoto H. The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor- α through nuclear factor- κ , and by 17 β -estradiol through Sp-1 in human vascular endothelial cells. *J Biol Chem* 2000;275:25781–90.
 19. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, et al. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 1995;96:1395–403.
 20. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NAD(P)H oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab* 2001;280:E685–94.
 21. Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21 (ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 1997;272:17810–14.
 22. Zill H, Günther R, Erbersdobler HF, Fölsch UR, Faist V. RAGE expression and AGE-induced MAP kinase activation in Caco-2 cells. *Biochem Biophys Res Commun* 2001;288:1108–11.
 23. Zhu P, Tang L, Zhao SC, Lu X, Hou FF, Fu N. Preparation of monoclonal antibodies against different epitopes at the extracellular domain of the human receptor for advanced glycation end product. *Di Yi Jun Yi Da Xue Xue Bao* 2004;24:129–32.
 24. Hou FF, Miyata T, Boyce J, Yuan Q, Chertow GM, Kay J, et al. β 2-Microglobulin modified with advanced glycation end products delays monocyte apoptosis. *Kidney Int* 2001;59:990–1002.
 25. Hou FF, Boyce J, Chertow GM, Kay J, Owen WF Jr. Aminoguanidine inhibits advanced glycation end products formation on beta2-microglobulin. *J Am Soc Nephrol* 1998;9:277–83.
 26. Tinsley JH, Wu MH, Ma W, Taulman AC, Yuan SY. Activated neutrophils induce hyperpermeability and phosphorylation of adherens junction proteins in coronary venular endothelial cells. *J Biol Chem* 1999;274:24930–4.
 27. Volberg T, Geiger B, Kartenbeck J, Franke WW. Changes in membrane-microfilament interaction in intercellular adherences junctions upon re-

- removal of extracellular Ca ions. *J Cell Biol* 1986; 102:1832–42.
28. Niggli V, Burger MM. Interaction of the cytoskeleton with the plasma membrane. *J Membr Biol* 1987;100:97–121.
 29. Madara J, Moore LR, Carlson S. Alterations of intestinal tight junction structure and permeability by cytoskeletal contraction. *Am J Physiol* 1987;253:C854–61.
 30. Otero K, Martinez F, Beltran A, Gonzalez D, Herrera B, Quintero G, et al. Albumin-derived advanced glycation end-products trigger the disruption of the vascular endothelial cadherin complex in cultured human and murine endothelial cells. *Biochem J* 2001;359:567–74.
 31. Schmidt AM, Yan SD, Yan SF, Stern DM. The biology of the receptor for advanced glycation end products and its ligands. *Biochim Biophys Acta* 2000;1498:99–111.
 32. Huttunen HJ, Fages C, Rauvala H. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J Biol Chem* 1999;274:19919–24.
 33. Widmann C, Gilson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999;79:143–80.
 34. Lee JC, Kassis S, Kumar S, Badger A, Adams JL. p38 mitogen-activated protein kinase inhibitors – mechanisms and therapeutic potentials. *Pharmacol Ther* 1999;82:389–97.
 35. Lander H, Tauras J, Ogiste J, Moss R, Schmidt AM. Activation of RAGE triggers a MAP kinase pathway regulated by oxidant stress. *J Biol Chem* 1997;272:17810–14.
 36. Ishihara K, Tsutsumi K, Kawane S, Nakajima M, Kasaoka T. The receptor for advanced glycation end-products (RAGE) directly binds to ERK by a D-domain-like docking site. *FEBS Lett* 2003;550:107–13.
 37. Klingenberg D, Gunduz D, Hartel F, Bindwald D, Schafer M, Piper HM, et al. MEK/MAPK as a signaling element in ATP control of endothelial myosin light chain. *Am J Physiol Cell Physiol* 2004;286:C807–12.
 38. Tinsley JH, Lanerolle PD, Wilson E, Ma W, Yuan SY. Myosin light chain kinase transference induces myosin light chain activation and endothelial hyperpermeability. *Am J Physiol Cell Physiol* 2000;279:C1285–9.
 39. Yuan Y, Huang QB, Wu HM. Myosin light chain phosphorylation: modulation of basal and agonist-stimulated venular permeability. *Am J Physiol* 1997;272:H1437–43.
 40. Guo ZJ, Hou FF, Zhang X, Liu ZQ, Wang L. Advanced glycation end products inhibit production of nitric oxide by human endothelial cells through activation of the p38 signal pathway. *Natl Med J China* 2002;82:1328–31.
 41. Kayyali US, Pennella CM, Trujillo C, Villa O, Gaestel M, Hassoun PM. Cytoskeletal changes in hypoxic pulmonary endothelial cells are dependent on MAPK-activated protein kinase MK2. *J Biol Chem* 2002;277:42596–602.
 42. Borbiev T, Birukova A, Liu F, Nurmukhambetova S, Gerthoffer WT, Garcia JN, et al. p38 MAP kinase-dependent regulation of endothelial cell permeability. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L911–8.