The Receptor for Advanced Glycation Endproducts Is Induced by the Glycation Products Themselves and TNF-α through NF-κB, and by 17β-estradiol through Sp-1 in Human Vascular Endothelial Cells

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Running title: *Mechanisms of RAGE gene induction in endothelial cells*
The binding of advanced glycation endproducts (AGE) to the receptor for AGE (RAGE) is known to deteriorate various cell functions and is implicated in the pathogenesis of diabetic vascular complications. Here we show that AGE, tumor necrosis factor-α (TNF-α) and 17β-estradiol (E₂) upregulated RAGE mRNA and protein levels in human microvascular endothelial cells and ECV304 cells, with the mRNA stability being essentially invariant. Transient transfection experiments with human RAGE promoter-luciferase chimeras revealed that the region from nucleotide number -751 to -629 and the region from -239 to -89 in the RAGE 5’-flanking sequence exhibited the AGE/TNF-α- and E₂-responsiveness, respectively. Site-directed mutation of an NF-κB site at -671 or of Sp-1 sites at -189 and -172 residing in those regions resulted in an abrogation of the AGE/TNF-α- or E₂-mediated transcriptional activation. Electrophoretic mobility shift assays revealed that ECV304 cell nuclear extracts contained factors which retarded the NF-κB and Sp-1 elements, and that the DNA-protein complexes were supershifted by anti-p65/p50 NF-κB and anti-Sp-1/estrogen receptor α antibodies, respectively. These results suggest that AGE, TNF-α and E₂ can activate the RAGE gene through NF-κB and Sp-1, causing enhanced AGE-RAGE interactions, which would lead to an exacerbation of diabetic microvasculopathy.
Glucose and other reducing sugars can react nonenzymatically with the amino groups of proteins to form reversible Schiff bases and, then, Amadori compounds. These early glycation products undergo further complex reactions to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation endproducts (AGE)\(^1\) (1). AGE are known to accumulate in various tissues at an extremely accelerated rate under a diabetic state, and are implicated in the development of diabetic vascular complications, e.g., retinopathy and nephropathy (1). We have shown previously that AGE exert their effects on endothelial cells and pericytes, the constituents of microvessels, through interactions with a cell-surface receptor for AGE (RAGE); AGE stimulate the growth of microvascular endothelial cells through an induction of vascular endothelial growth factor (VEGF) leading to angiogenesis on the one hand (2), and inhibit prostacyclin production and stimulated plasminogen activator inhibitor-1 synthesis by the endothelial cells on the other (3). AGE exhibit a growth inhibitory action on pericytes (4), which would lead to pericyte loss, the earliest histological hallmark in diabetic retinopathy (5).

RAGE belongs to the immunoglobulin superfamily of cell surface molecules (6, 7). It is expressed in multiple tissues (8) and interacts with various ligands including AGE (9, 10). The engagement of RAGE by AGE has been reported to induce cellular oxidant stress, activating the transcription factor nuclear factor-κB (NF-κB) (11, 12), resulting in the perturbation of a variety of vascular homeostatic functions (9, 10). AGE-RAGE interaction thus has been thought to play a central role in the development of diabetic vasculopathy. To determine how the RAGE gene is regulated under a diabetic state is, therefore, important for clarifying the pathogenesis of diabetic complications as well as for understanding the physiological roles of RAGE.

It has been reported that AGE-rich blood vessels show enhanced RAGE immunoreactivity (13); this implies the possibility that AGE themselves may up-regulate the RAGE expression. Among cytokines, tumor necrosis factor-α (TNF-α) is thought to be involved in the development of diabetes (14). Evidence has accumulated that serum TNF-α
levels are increased in non-insulin-dependent diabetes mellitus (NIDDM) (15-17) and that TNF-α can activate the NF-κB pathway (18-20). Recently, NF-κB has been reported to play a role in the basal and lipopolysaccharide (LPS)-induced expression of the RAGE gene (21). It has also been reported that diabetic vasculopathy is often aggravated during pregnancy, probably due to the increased level of serum estrogen (22-24). From these observations, the possibility that TNF-α and estrogen worsen the diabetic complications through the induction of RAGE gene expression should also be considered.

In the present study, we thus examined the effects of AGE, TNF-α and 17β-estradiol (E₂) on RAGE gene expression and found that the three agents are capable of up-regulating the RAGE mRNA and protein levels in human microvascular endothelial cells, but not nonglycated BSA, other cytokines and an antiestrogen did not affect on the RAGE mRNA levels. mRNA stability and promoter assays demonstrated that the induction was at the transcriptional level, and that AGE and TNF-α induced the RAGE gene through an activation of NF-κB while E₂ induced the gene through Sp-1.
EXPERIMENTAL PROCEDURES

Materials --- Bovine serum albumin (BSA) (fraction V, fatty acid-free, endotoxin-free) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). TNF-α was purchased from Becton Dickinson Labware (Bedford, MA). Transforming growth factor-β1 (TGF-β1) and interferon-γ (IFN-γ) were from R&D Systems, Inc. (Minneapolis, MN). E₂ and 4-hydroxytamoxifen (4-OH tamoxifen) were from Sigma Chemical Co. (St. Louis, MO). [γ-³²P]ATP (6,000 Ci/mmol) was from DuPont NEN (Boston, MA). Restriction enzymes and T₄ polynucleotide kinase were from TAKARA (Kyoto, Japan). Rabbit polyclonal antibodies raised against recombinant human RAGE extracellular domain (amino acids 24 to 321) (7) were kindly provided by the Institute of Biological Science, Mitsui Pharmaceutical Inc (Mobara, Japan).

Cells --- Human skin microvascular endothelial cells (HMVEC) (Cascade Biologics, Inc., Portland, OR) were maintained in HuMedia-EB2 supplemented with 5% fetal bovine serum (FBS), gentamycin (50 µg/ml), amphotericin B (50 ng/ml), basic fibroblast growth factor (5 ng/ml), heparin (10 µg/ml), epidermal growth factor (10 ng/ml) and hydrocortisone (1 µg/ml) according to the supplier’s instructions (Kurabo Corp., Osaka, Japan) in humidified incubators containing 5% CO₂. The human umbilical vein endothelial cell (HUVEC)-derived cell line ECV304 (25) was kindly donated by Dr. Yoshio Sawasaki, National Defense Medical College, Tokorozawa, Japan, and was maintained in M199 (Nissui Pharmaceutical Co., LTD, Tokyo, Japan) supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). All experiments using HMVEC and ECV304 cells were carried out in media lacking epidermal growth factor and hydrocortisone, and FBS, respectively. These media are referred to as the experimental media.

Preparation of AGE-BSA --- AGE-BSA was prepared by incubating BSA with 0.5 M glucose at 37°C for 6 weeks under sterile conditions as described previously (2). After unincorporated sugars were removed by dialysis against phosphate-buffered saline (PBS), glucose-modified higher-molecular weight materials were used as AGE-BSA. Control non-glycated BSA was incubated under the same conditions except for the absence of glucose.
The concentration of AGE-BSA and control BSA were determined by the method of Bradford (26).

**Measurement of RAGE mRNA by Quantitative Reverse Transcription-PCR (RT-PCR)**

*Method* --- Poly(A)^+^RNAs were isolated from subconfluent cultures of HMVEC or ECV304 cells incubated under various conditions, using a Quick prep micro mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and analyzed by RT-PCR with a GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA) as previously described (27). Oligodeoxyribonucleotide primers and probes for human RAGE, VEGF, and β-actin mRNA were the same as described previously (4, 28). The amounts of poly(A)^+^RNA templates and cycle numbers for amplification were chosen in quantitative ranges where reactions proceeded linearly as described previously (28, 29); 30 ng of templates and 30 cycles were chosen for amplifying human RAGE mRNA, 30 ng and 40 cycles for human VEGF mRNA, and 30 ng and 20 cycles for β-actin mRNA. The fragments amplified with PCR were sequence-verified on both strands by the chain termination method (30). Five µl aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and transferred to a Hybond-N^+^ nylon membrane (Amersham Pharmacia Biotech), and the membrane was hybridized with the respective probes which had been 32P-end-labeled with polynucleotide kinase (27). Signal intensities of hybridized bands were measured by a BAS 1000 BioImage analyzer (Fuji Photo Film Co. Ltd., Hamamatsu, Japan).

**Analysis of RAGE mRNA Stability** --- HMVEC or ECV304 cells were treated with TNF-α, E2 or AGE-BSA for 4 h, and further cultured in the presence of 10 µg/ml of actinomycin D (Sigma Chemical Co.) for various time periods. Total RNAs were isolated from the cultures with Isogen (Nippon Gene, Toyama, Japan) according to the method described by Chomczynski *et al.* (31), and analyzed by the quantitative RT-PCR method described above. The amounts of total RNA templates and cycle numbers for amplification were chosen in quantitative ranges (29); 300 ng of templates and 35 cycles were chosen for amplifying human RAGE mRNA, and 300 ng and 20 cycles for β-actin mRNA.

**Western Blot Analysis** --- Subconfluent cultures of HMVEC or ECV304 cells were
incubated with TNF-α, E₂ or AGE-BSA for 24 h. After the incubation, cells were washed with cold PBS, scraped off in cold PBS, and pelleted by centrifugation at 300 x g at 4°C for 5 min. The cells were lysed immediately by sonication in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and boiled at 95°C for 5 min. Twelve µg of the cell lysates were resolved by SDS-PAGE (12.5%), and then transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was treated with the anti-RAGE polyclonal antibodies, and the immunoreactive bands were visualized with an ECL detection system (Amersham Pharmacia Biotech) as described previously (32). Signal intensities of the resultant bands were determined by densitometry using BIO-PROFIL 1-D (Ver. 5.08) software (Vilber Lourmat Biotechnology, Marne La Vallée, France).

**Construction of the RAGE Promoter-Luciferase Chimeras ---** The chimeric genes for transfection experiments were constructed by ligating the 5’-flanking regions of differing lengths of the human RAGE gene upstream of the luciferase gene in a pGL3-basic vector (Promega Corp., Madison, WI) (See Fig. 5A). The genomic DNA fragments of the human RAGE gene were amplified by PCR using a cosmid named KS71 (33) as a template, which was kindly provided by Professor Toshimichi Ikemura (National Institute of Genetics, Shizuoka, Japan). The PCR primers employed in the amplification reactions are shown in Table I. Furthermore, we constructed two additional chimeric genes. The DNA fragments containing exon 1 to 11 plus intron 1 to 10 or the 3’-flanking region of the human RAGE gene were amplified by PCR using the cosmid KS71 and specific primers shown in Table I, and were ligated to downstream of the luciferase gene in the pGL3-Basic vector that had carried the longest fragment of the 5’-flanking region (See Fig. 5A). All the fragments obtained were sequence-verified.

**Site-directed Mutagenesis ---** Mutations were introduced into the RAGE promoter-luciferase chimeras using a GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega) according to the manufacturer’s protocol. The nucleotide sequences of the
mutagenic oligodeoxyribonucleotide primers were 5’-ACTGTCAGAGTTGGTCCCCCTCCCATTAAG-3’ (nucleotides -683 to -652), 5’-GTGACTGTACCAGA ATCTGGTAGTACCCAGG-3’ (-202 to -171), 5’-CTGGTAGTACCCAGGAATGGGGTGATAATTAT-3’ (-185 to -154), and 5’-ACTGTACCAGA ATCTGGTAGTACCCAGGAATGGGGTGATAATTAT-3’ (-199 to -157) for yielding pGL-5 NF-κB#2m, pGL-7 Im, pGL-7 IIm or pGL-7 ImIIm, respectively (mutated sites are indicated by underlines) (See schematic representations in Fig. 6A.). pGL-5 NF-κB#2m contained the region -751 to +43, but with the mutation in an NF-κB site (-671 to -663). pGL-7 Im and pGL-7 IIm contained the region -239 to +43, but with the mutations in one of two Sp-1 sites (-189 to -181 and -172 to -166), respectively. pGL-7 ImIIm had the mutations in both Sp-1 sites. All the mutated constructs were sequence-verified.

Transfection Experiments and Luciferase Assay --- ECV304 cells or HMVEC (2 x 10^5 cells) were plated into the wells of 6-well tissue culture plates (Becton Dickinson Labware) one day before transfection. For ECV304 cells, the DNA/cationic lipid mixture for transfection was composed of test plasmid (2 µg), pRL-SV40 vector (0.5 µg) (Promega) that served as an internal control to normalize luciferase activities, TransFast™ Transfection Reagent (7.5 µl) (Promega), and 1ml of the experimental medium. Cells were exposed to the DNA/cationic lipid mixture for 2 h, then received 5 ml of the FBS-containing medium and were further incubated for 36 h at 37°C. For transfection of HMVEC, test plasmid (2 µg) and pRL-CMV vector (1 µg) (Promega) were added to a tube containing 100 µl of Opti-MEM 1 (Life Technologies, Inc., Rockville, MD). Lipofectin reagent (6 µl) (Life Technologies, Inc.) was added to another tube containing 100 µl of Opti-MEM 1. The plasmid DNA and Lipofectin reagent were then mixed together. After incubation at room temperature for 30 min, DNA-liposome complex was diluted with 800 µl of Opti-MEM 1. Cells were exposed to the mixture for 8 h, then received 2 ml of the FBS-containing medium and were further incubated for 36 h at 37°C. After the incubation, the cells were treated with TNF-α, E₂ or AGE-BSA for 8 h in the experimental medium. Luciferase activities were measured using a
Dual-Luciferase™ Reporter Assay System (Promega) according to the manufacturer’s protocol with a luminometer (Fluoroskan Ascent FL Ver. 2. 2. 4, Labsystems, Helsinki, Finland).

Preparation of Nuclear Extracts from Cultured Cells --- Nuclear extracts were prepared essentially as described by Schreiber et al. (34). Briefly, ECV304 cells (2 x 10⁶) were plated onto 75-cm² tissue culture flasks (Becton Dickinson Labware) in the complete medium and left for 24 h at 37°C. The cells were further incubated in the experimental medium at 37°C for 12 h and then treated with TNF-α, E₂ or AGE-BSA for 4 h. After the treatment, the cells were washed twice with ice-cold PBS, scraped off in PBS (10 ml) and pelleted by centrifugation at 3,000 rpm in a Beckman GH3.7 roter at 4°C for 5 min. The pelleted cells were resuspended in 0.4 ml of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and placed on ice for 15 min. After the addition of 25 µl of 10% Nonidet P-40, the suspension was vortexed for 15 s and centrifuged at 13,000 rpm in a HITACHI T15S roter at 4°C for 30 min. The resultant nuclear pellets were washed with buffer A and resuspended in 0.1 ml of a solution containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride by constant agitation for 15 min at 4°C. The nuclear lysates were centrifugated at 13,000 rpm at 4°C for 10 min, and the supernatants were collected as nuclear extracts. The protein concentrations of the nuclear extracts were determined by the method of Bradford (26).

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay --- The wild type and mutant double-stranded oligodeoxyribonucleotides encompassing the NF-κB site (nucleotide numbers -671 to -663) or the two Sp-1 sites (-189 to -181 and -172 to -166) were prepared. Their sequences were 5’-AGAGTGGGGAACCCCTCCCA-3’ and 5’-AGAGTGGGGTTCCCCTCCCA-3’ (-677 to -658) as the wild and mutated NF-κB elements, respectively, and 5’-CCCAGAGGCTGGTAGTACCCAGGGGTGGGTA-3’ and 5’-CCCAGAAAC TGGTAGTACCCAGGAATGGGGTGA-3’ (-193 to -161) as the wild and mutated Sp-1 elements, respectively (mutated sites are indicated by underlines). Twenty-five
fmol of wild-type oligodeoxyribonucleotides, which had been \(^{32}\)P-end-labeled with polynucleotide kinase (27), were incubated with 5 µg of nuclear extracts at room temperature for 30 min. Samples were then loaded onto 6% polyacrylamide gels and run in 0.2 x Tris-borate/EDTA electrophoresis buffer at 10 V/cm for 2-3 h. The gels were dried and autoradiographed at -80°C overnight. For competition assay, nuclear extracts were first incubated with a 50-fold excess of unlabeled wild-type or mutant oligodeoxyribonucleotides at room temperature for 15 min, and then incubated with the labeled wild-type probe for 30 min under the same conditions as described above. For supershift assays, antibodies to NF-κB p65, NF-κB p50, Sp-1, estrogen receptor (ER) α or ERβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the nuclear extracts and incubated at 4°C for 12 h. The antibody-treated nuclear extracts were subsequently incubated with the labeled oligodeoxyribonucleotides for 30 min under the same conditions as described above.

Statistical Analysis --- Paired t-tests and one-way analysis of variance (ANOVA) with Tukey's range tests were used to test for significant differences between groups. All experiments were carried out at least three times.
RESULTS

*TNF-α, 17β-estradiol (E₂) and AGE-BSA Increased the RAGE mRNA and Protein Levels in Human Microvascular Endothelial Cells (HMVEC) and ECV304 Cells* --- To examine the effects of TNF-α, E₂ and AGE-BSA on the RAGE mRNA level in HMVEC, poly(A)+RNAs were isolated from cells which had been exposed to various concentrations of these agents for 4 h, and analyzed by the quantitative RT-PCR method. As shown in Fig. 1A, TNF-α, E₂ and AGE-BSA increased the RAGE mRNA levels in dose-dependent manners. The extents of induction and the peak concentrations were about 3-fold at 100 ng/ml TNF-α, 10 nM E₂ and 50 µg/ml AGE-BSA, respectively, when normalized by β-actin mRNA-derived signals used as an internal control. Next, we examined the time course of the RAGE mRNA induction. HMVEC were treated for various time periods with TNF-α, E₂ and AGE-BSA at their most effective doses. As shown in Fig. 1B, the mRNA levels began to increase at 2 h and reached a maximum at ∼8 h after the addition of either of the three agents. On the other hand, exposure of HMVEC to TGF-β1 (10 ng/ml), IFN-γ (165 ng/ml), and non-glycated BSA (50 µg/ml) for 4 h did not affect the RAGE mRNA levels (Fig. 1C). 4-OH tamoxifen (10 nM), an antiestrogen, abolished the E₂-induced RAGE mRNA induction (Fig. 1C).

We next determined the RAGE mRNA levels in ECV304 cells, an immortalized cell line derived from human umbilical vein endothelial cells (25). This cell line exhibited the same responsiveness to AGE-BSA with respect to VEGF induction (Fig. 2A) as did HMVEC (2), the most effective dosage of AGE-BSA being 50 µg/ml in both cultures. The extent of induction was about 4-fold. When exposed to TNF-α (100 ng/ml), E₂ (10 nM) or AGE-BSA (50 µg/ml) for 4 h, the RAGE mRNA levels in the ECV304 cells were also increased about 2-fold compared with those in the control unexposed cells (Fig. 2B).

We next examined whether the increase in RAGE mRNA was actually followed by an increase in RAGE proteins in HMVEC and ECV304 cells. The cells were treated with TNF-α, E₂ or AGE-BSA for 24 h, and subjected to Western blot analysis with anti-RAGE polyclonal antibodies. As shown in Fig. 3, one major immunoreactive band was marked at 46
kDa in either HMVEC (Fig. 3A) or ECV304 cells (Fig. 3B), being consistent with our previous report (3), and its intensity was increased by the treatment with the three agents. The RAGE protein levels in TNF-α- (lane 3), E₂- (lane 4) or AGE-BSA- (lane 5) treated HMVEC and ECV304 cells were about 2-fold higher than the basal levels. The results indicated that ECV304 cells retained the ability to respond to those agents as did primary cultured endothelial cells, and subsequently we used this cell line to examine the mechanisms of RAGE gene induction.

Effects of TNF-α, E₂ and AGE-BSA on RAGE mRNA Stability in HMVEC and ECV304 Cells --- We next determined the RAGE mRNA stability in HMVEC and ECV304 cells exposed or not exposed to the three agents to determine which step of gene expression accounted for the increase in RAGE mRNA levels. The cells were incubated in the presence or absence of TNF-α, E₂ or AGE-BSA for 4 h, then incubated with actinomycin D for various time periods, and underwent quantitative RT-PCR analyses. As shown in Fig. 4A and B, the half lives of RAGE mRNA in TNF-α-, E₂- and AGE-BSA-treated or not treated HMVEC and ECV304 cells were calculated from the RAGE and β-actin mRNA-derived signals to be between 2.1 and 2.8 h, and there was no statistically significant difference among them. The results suggested that the TNF-α-, E₂- and AGE-BSA-induced increase in RAGE mRNA was achieved at the transcriptional level.

Identification of the cis-Elements Responsive to TNF-α, E₂ and AGE-BSA in the RAGE Promoter --- To confirm whether TNF-α, E₂ and AGE-BSA did induce the RAGE gene transcription and, if so, to delimit the regions involved in such transcriptional activations, a series of chimeric 5′-deletion promoter-luciferase reporter constructs were prepared. Schematic representations of the constructs are shown in Fig. 5A. ECV304 cells were transiently transfected with the constructs, and the effects of TNF-α, E₂ and AGE-BSA on the luciferase activity in the transfected cells were determined. pGL-1 carried the longest 5′-flanking region of the RAGE gene (1689 bp upstream of the transcription start site (14)), and when the pGL-1-transfected cells were exposed to TNF-α (Fig. 5B), E₂ (Fig. 5C) or AGE-BSA (Fig. 5D), the promoter activities (closed columns) increased significantly (1.5−
2-fold) compared with those in unexposed cells (open columns). The same concentration of non-glycated BSA did not induce the luciferase activity in the transfected cells (data not shown). Deletion of the 5′-flanking region of the RAGE gene to -751 (pGL-2 to -5) did not affect the TNF-α- and AGE-induced luciferase expression, but deletion to -629 (pGL-6) abolished the induction (Fig. 5B and D). In contrast, the construct with a deletion to -239 (pGL-7) still retained the E2-induced luciferase expression but deletion to -89 (pGL-8) abolished the induction (Fig. 5C). The -751 to -629 region contained an NF-κB site (-671 to -663) and the -239 to -89 region contained two Sp-1 sites (-189 to -181 and -172 to -166). The results thus suggested that the NF-κB site and the Sp-1 sites might be required for the TNF-α/AGE- and E2-induced activation of the RAGE gene promoter, respectively.

We also tested additional constructs that carried genomic fragments containing exon 1 to 11 and intron 1 to 10 or the 3′-flanking region (pGL-e1-11 and pGL-3′fl, respectively) in addition to the pGL-1 5′-promoter region. As shown in Fig. 5E, when the pGL-e1-11- or pGL-3′fl-tranfected cells were stimulated by TNF-α (closed columns), E2 (lattice columns) or AGE-BSA (dotted columns), the extents of luciferase induction were almost indistinguishable from those in the pGL-1-transfected cells. The results indicated that there were neither stimulatory nor silencing elements in the exon/intron or 3′-flanking region of the RAGE gene which could affect its responsiveness to TNF-α, E2 or AGE-BSA.

To determine the role of the NF-κB site at -671 to -663 in the TNF-α and AGE activation of the RAGE promoter, site-directed mutagenesis was performed at that site (pGL-5 NF-κB#2m) (Fig. 6A). When luciferase activities were assayed in cells transfected with the mutant, the inducibility by both TNF-α and AGE-BSA was found to be totally abolished (Fig. 6B). Similarly, we also determined the role of the two Sp-1 sites at -189 to -181 and -172 to -166 in the E2-dependent transcriptional activation. We altered each of the two Sp-1 sites (pGL-7 Im and pGL-7 IIm) or both sites (pGL-7 ImIIm) (Fig. 6A), and the E2 effect on luciferase activities in the mutant transfectants was tested (Fig. 6C). The E2 inducibility of luciferase activities was decreased in pGL-7 Im- and pGL-7 IIm-transfected cells and totally abolished in pGL-7 ImIIm-transfected cells. The results indicated that the
NF-κB binding site and the two Sp-1 binding sites were essential for the TNF-α/AGE- and E₂-induced activation of the RAGE gene, respectively.

**TNF-α/AGE-BSA and E₂ Induced the Nuclear Protein-DNA Complex Formation on the NF-κB and Sp-1 Sites** --- To further characterize the roles of the NF-κB and Sp-1 sites in the regulation of RAGE gene expression, we conducted EMSA using synthetic oligodeoxyribonucleotides corresponding to the putative NF-κB binding site and to the two Sp-1 binding sites, and nuclear extracts (5 µg) from ECV304 cells. As shown in Fig. 7, the extracts from cells treated with TNF-α (A, lane 2, arrow) and AGE-BSA (B, lane 2, arrow) exhibited increased binding activities to the NF-κB site compared with those from untreated cells (A and B, lane 1). In E₂-treated cells, the binding activities to the Sp-1 sites also increased compared with the basal conditions (Fig. 7C). In each case, the specificity of the gel-retarded band was demonstrated by competition with a 50-fold excess of unlabeled wild-type or mutant oligodeoxyribonucleotides. Viz., preincubation with wild-type probes resulted in decreased binding to the NF-κB site (Fig. 7A and B, lane 3) and the Sp-1 sites (Fig. 7C, lane 3). In contrast, preincubation with mutant probes had little effect on the binding to the NF-κB and Sp-1 sites (Fig. 7A, B and C, lane 4).

Next, we examined by supershift assays which members of the NF-κB family were responsible for the stimulation of the RAGE gene expression by TNF-α and AGE-BSA. When the nuclear extracts from the cells exposed to TNF-α (Fig. 7D) or AGE-BSA (Fig. 7E) were incubated with either anti-p65 or anti-p50 antibody, a more slowly migrating band (Fig. 7D and E, arrow) newly appeared with a concomitant decrease in the original complex (Fig. 7D and E, closed arrowhead). We also examined which factors were involved in the E₂ induction of the RAGE gene. When the nuclear extracts from the cells treated with E₂ (Fig. 7F) were incubated with either anti-Sp-1 or anti-ERα antibody, the original band DNA-protein complex was supershifted upward (Fig. 7F, lanes 2 and 3), whereas anti-ERβ antibody gave no effect (Fig. 7F, lane 4). The results indicated that the DNA-binding complex for the NF-κB site was composed mainly of p50 and p65 members of the NF-κB family, and that the DNA-binding complex for the Sp-1 sites was composed mainly of Sp-1 and ERα.
The NF-κB and Sp-1 Sites Also Mediated the TNF-α/AGE-BSA- and E₂-Induced RAGE Gene Expression in HMVEC ---

To confirm whether the NF-κB site and Sp-1 sites are also involved in the TNF-α/AGE-BSA- and E₂-induced upregulation of RAGE gene in normal diploid microvascular endothelial cells, we performed luciferase assays in HMVEC using pGL-5 and its mutants for the TNF-α/AGE-BSA-induced transcriptional activation, and pGL-7 and its mutants for the E₂-induced activation. As shown in Fig. 8A, TNF-α and AGE-BSA induced luciferase expression ∼ 3-fold in pGL-5-transfected cells, the extent of induction being comparable with those of the RAGE mRNA induction in HMVEC (Fig. 1). The inducibility by both TNF-α and AGE-BSA was found to be totally abolished in pGL-5 NF-κB#2m- or pGL-6-transfected cells (Fig. 8A). E₂ induced luciferase expression about 2.6-fold in pGL-7-transfected cells (Fig. 8B). This extent of induction was also comparable with that of the RAGE mRNA induction by E₂ in HMVEC (Fig. 1). The E₂ inducibility of luciferase activities was totally abolished in pGL-7 ImIIm- or pGL-8-transfected cells (Fig. 8B).
DISCUSSION

We (2-4, 35) and others (36-39) have shown previously that interactions between AGE and RAGE cause phenotypic changes in microvascular endothelial cells and pericytes that are characteristic of diabetic vasculopathy. In this paper, we have demonstrated for the first time that AGE, the RAGE ligand itself, TNF-α and E₂ specifically up-regulate RAGE mRNA and protein levels in human vascular endothelial cells, and that this process is mediated by two distinct nuclear complexes, viz. p65/p50 NF-κB and Sp-1/ERα.

It has been reported that AGE-rich vasculature exhibits enhanced RAGE immunoreactivity in the sites of diabetic microvascular injury (13). This finding suggested that AGE themselves could activate the RAGE gene expression, and that increased RAGE might further transduce AGE signals within microvascular cells. The present study has clearly shown that AGE-BSA did, but non-glycated BSA did not, up-regulate the RAGE gene expression and that this was achieved at the level of transcription, because RAGE mRNA half-lives were unchanged by the treatment with AGE (Fig. 4), and because AGE elicited the expression of the reporter gene linked to the 5’ promoter region of the RAGE gene (Fig. 5). The experiments with the RAGE promoter-reporter gene constructs demonstrated the presence of an AGE-responsive element in the -751 to -629 region of the human RAGE gene, and that an NF-κB site residing at -671 conferred the responsiveness to AGE on the RAGE gene (Figs. 5, 6 and 8). Further, the AGE-dependent formation of the NF-κB element-p65/p50 complex was demonstrated (Fig. 7). These results are considered to be consistent with the recent observations by others (11, 12) that AGE engagement of RAGE induces cellular oxidant stress, thereby activating the transcription factor NF-κB. The fact that AGE themselves induce the RAGE gene appears to be important when considering the mechanisms of development of diabetic vascular complications. Such a positive feedback loop in the diabetic state may exacerbate diabetic vasculopathy, exemplified by retinopathy and nephropathy.

We also demonstrated that TNF-α is another inducer of the RAGE gene (Figs. 1-3). TNF-α is known to be overexpressed in adipose tissue under obese and diabetic states (15-
17) and to cause insulin resistance, the central and early component of NIDDM (18). TNF-α affects not only insulin sensitivity by suppressing insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (40) but also cell survival by NF-κB activation (20-22). We thus propose that an increased TNF-α level in NIDDM patients may worsen diabetic vasculopathy via RAGE gene induction. The TNF-α-induced stimulation of human RAGE gene was found to be also transcriptional and to be achieved by the same NF-κB element and binding complex (Figs. 5-8) as was the AGE stimulation. Recently, Li et al. (14) reported that the RAGE gene was activated by LPS via NF-κB sites at -671 and -467 in bovine aortic endothelial cells and rat vascular smooth muscle cells, and that both sites were required for full activation by LPS. Our results indicated that only one NF-κB site at -671 was required for RAGE gene induction by AGE-BSA and TNF-α in human microvascular endothelial cells (Figs. 5, 6 and 8). The discrepancy may be due to differences in the species- or ligand-specificity of the gene activation. The results suggest that the factors which have the ability to activate the NF-κB pathway can induce RAGE gene expression and have the potential to aggravate the AGE-mediated diabetic complications.

Pregnancy is known to worsen diabetic retinopathy (22-24). Schocket et al. (41) showed that a decrease in the retinal volumetric blood flow in diabetic patients during pregnancy might exacerbate retinal ischemia and aggravate retinopathy. Suzuma et al. (42) demonstrated that E2 at the concentration often observed during pregnancy (∼10 nM) stimulates VEGF-dependent angiogenesis through the up-regulation of VEGF receptor-2 expression. However, the mechanisms underlying the adverse effects of E2 on diabetic complications are not yet fully understood. In this study, we demonstrated that E2 is an alternative inducer of the RAGE gene in human endothelial cells, enhancing its transcription at the concentration of 10 nM (Figs. 1-3). An antiestrogen, 4-OH tamoxifen, totally abolished the E2-induced RAGE mRNA induction in HMVEC, while itself caused no change in the RAGE mRNA level (Fig. 1C). This was regarded as an indication that E2 may act on RAGE gene through an estrogen receptor. The RAGE promoter does not contain any classical estrogen-responsive element (ERE) (43) but contains several GC-rich boxes which
can bind to the transcription factor Sp-1 (14, 33, 44). Clearly, E₂ utilizes a device that is different from those employed by AGE and TNF-α in the induction of RAGE gene. Two Sp-1 binding sites at -189 and -172 and an Sp-1/ER complex were involved in the E₂ activation, and full E₂ responsiveness required both cis-acting elements (Figs. 5-8). Recent studies from other laboratories have shown that an interaction of Sp-1/ER complex with GC-rich motifs in the promoter region is required for the transcriptional activation of several E₂-responsive genes (45-49). The results obtained in this study suggest that the E₂ induction of RAGE may partly underly the exacerbation of diabetic retinopathy during pregnancy.

There are many genes that are regulated by the combination of NF-κB and Sp-1, and in some cases a direct interaction between NF-κB protein and Sp-1 protein has been demonstrated (50, 51). In the induction of the RAGE gene, however, such a direct interaction between the two factors would seem unlikely to occur because the Sp-1-mediated E₂ responsiveness was still retained in the constructs with the deletion of the more than 500-nucleotide region encompassing the NF-κB element (Fig. 5).

Cytokines that did not affect the endothelial cell expression of RAGE gene included TGF-β1 and IFN-γ (Fig. 1C). This indicated that Smad (52) or JAK-STAT (53) systems would not be involved in the regulation of RAGE gene.

In summary, we found that AGE and TNF-α enhanced RAGE expression through activation of the p65/p50 complex of NF-κB, and that E₂ also activated RAGE expression through activation of the Sp-1/ER complex. Though the extent was rather modest, the three agents consistently increased the RAGE mRNA and protein levels in vascular endothelial cells. Chronic exposure to AGE, TNF-α and/or E₂ and sustained enhancement of RAGE expression may cause a further accumulation of AGE in the vasculature, resulting in an exacerbation of AGE-RAGE-mediated vascular dysfunctions. Such mechanisms of RAGE gene activation probably have evolved to regulate functions of this multiligand receptor in various biologic processes, such as neurite formation of cortical neurons mediated by amphoterin engagement (54) and proinflammatory responses mediated by a recently
discovered endogenous ligand, EN-RAGE (55). Obviously, diabetes abuses the molecular devices for the RAGE gene regulation, resulting in the formation of a vicious circle which may eventually lead to the development and progression of diabetic vasculopathy. The enhanced interaction between AGE and RAGE may further increase VEGF expression in endothelial cells and/or retinal pigment epithelial cells (2, 56), resulting in angiogenesis, and also inhibit pericyte growth, leading to pericyte loss (4). Although more studies are needed to better clarify the significance of the RAGE gene activation by AGE, TNF-α and E₂ as well as immediate post-RAGE signaling events that lead to NF-κB and Sp-1 activations, inhibition of the RAGE gene activation may become a promising target for the prevention and treatment of diabetic vascular complications.

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FOOTNOTES

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¹Abbreviations used are: AGE, advanced glycation end products; RAGE, receptor for AGE; VEGF, vascular endothelial growth factor; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-alpha; NIDDM, non-insulin-dependent diabetes mellitus; LPS, lipopolysaccharide; E₂, 17β-estradiol; BSA, bovine serum albumin; TGF-β1, transforming growth factor-beta1; IFN-γ, interferon-gamma; HMVEC, human skin microvascular endothelial cells; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; IRS-1, insulin receptor substrate-1; ERE, estrogen-responsive element.
FIGURE LEGENDS

FIG. 1. Effects of TNF-α, 17β-estradiol (E₂) and AGE-BSA on RAGE mRNA levels in human skin microvascular endothelial cells (HMVEC). (A) HMVEC were incubated for 4 h with the indicated concentrations of TNF-α, E₂, or AGE-BSA. Poly(A)+RNAs were then isolated and analyzed by RT-PCR as described under EXPERIMENTAL PROCEDURES. (B) HMVEC were treated with TNF-α (100 ng/ml), E₂ (10 nM) or AGE-BSA (50 µg/ml) for the indicated time periods, and assayed for RAGE mRNA levels. (C) HMVEC were treated with TNF-α (100 ng/ml = 5 x 10³ U/ml), TGF-β1 (10 ng/ml), IFN-γ (165 ng/ml = 5 x 10³ U/ml), E₂ (10 nM), 4-OH tamoxifen (10 nM), E₂ plus 4-OH tamoxifen, AGE-BSA (50 µg/ml), or non-glycated BSA (50 µg/ml) for 4 h, and assayed for RAGE mRNA levels. The TGF-β1 and IFN-γ concentrations employed were those that caused near maximal biological effects (57, 58). Graphs indicate the quantification of the RAGE mRNA levels. Intensities of the RAGE mRNA signals were normalized with those of β-actin mRNA-derived signals, and are related to the value of the control without additives. Columns and Bars indicate means and standard deviations, respectively, in three independent experiments. *, p<0.05; **, p<0.01 (vs. control).

FIG. 2. AGE-induction of VEGF mRNA and effects of TNF-α, 17β-estradiol (E₂) and AGE-BSA on RAGE mRNA levels in ECV304 cells. (A) ECV304 cells were treated with the indicated concentrations of AGE-BSA for 4 h, and then assayed for the VEGF mRNA levels by RT-PCR. Closed and open columns indicate signals derived from VEGF₁₂₁ and VEGF₁₆₅, respectively. Graph indicates the quantification of the VEGF mRNA levels. Intensities of the VEGF mRNA signals were normalized with those of β-actin, and are related to the value of the control. (B) ECV304 cells were treated with TNF-α (100 ng/ml), E₂ (10 nM) or AGE-BSA (50 µg/ml) for 4 h, and assayed for the RAGE mRNA levels. Graph indicates the quantification of the RAGE mRNA levels. Intensities of the RAGE mRNA signals were normalized with those of β-actin mRNA-derived signals, and are related to the value of the control. Columns and Bars indicate means and standard deviations, respectively,
in three independent experiments. *, p<0.05; **, p<0.01 (vs. control).

FIG. 3. RAGE protein levels. (A) HMVEC and (B) ECV304 cells were incubated in the presence of TNF-α (100 ng/ml), E₂ (10 nM) or AGE-BSA (50 µg/ml) for 24 h, and then lysed in SDS-PAGE sample buffer. Twelve µg of protein per lane were run on SDS-12.5% polyacrylamide gel under reducing conditions, and subjected to immunoblot analysis using anti-RAGE polyclonal antibody as described under EXPERIMENTAL PROCEDURES. rRAGE, 50 ng of recombinant human RAGE extracellular domain expressed in Escherichia coli. Control, untreated cells. Graphs depict quantitative representations of RAGE protein levels in (A) HMVEC and (B) ECV304 cells. Data were related to the value of the respective controls. Columns and Bars indicate means and standard deviations, respectively, in three independent experiments. *, p<0.05; **, p<0.01 (vs. control).

FIG. 4. RAGE mRNA stability. (A) HMVEC and (B) ECV304 cells were treated with TNF-α (100 ng/ml), E₂ (10 nM) or AGE-BSA (50 µg/ml) for 4 h, and further cultured in the presence of 10 µg/ml actinomycin D for the indicated time periods. RAGE mRNA levels were determined by RT-PCR with total RNAs extracted from the cells. Control, cultures without the additives but with actinomycin D. Graphs depict quantitative representations of the RAGE mRNA levels in (A) HMVEC and (B) ECV304 cells. Data were normalized by β-actin mRNA-derived signals. Essentially the same results were obtained in three independent experiments.

FIG. 5. Construction of RAGE promoter luciferase-chimeras and identification of TNF-α, 17β-estradiol (E₂) or AGE-BSA responsive regions. (A) Schematic representation of the 5’-deleted RAGE promoter-luciferase reporter fusion gene constructs. Transcription start site (14) is designated as +1. Numbers in parentheses indicate nucleotide positions 5’ to the transcription start site. Relative luciferase activities in ECV304 transfectants treated with TNF-α (100 ng/ml) (B), E₂ (10 nM) (C) and AGE-BSA (50 µg/ml)
(D). *Open and closed columns* indicate the mean values of untreated and treated cells, respectively. Data were normalized by pRL-SV40-derived luciferase activities used as an internal control, and related to the value of untreated pGL-1-transfected cells. *Bars*, standard deviations of nine independent experiments. (E) Relative luciferase activities in ECV304 cells transiently transfected with pGL-1, pGL-e1-11, or pGL-3’fl in the absence (*open columns*) or presence of TNF-α (100 ng/ml) (*closed columns*), E₂ (10 nM) (*lattice columns*) or AGE-BSA (50 µg/ml) (*dotted columns*). Data were normalized by the activities derived from the internal control. Values are shown as the mean ± SD of four independent experiments. Statistical analysis was performed using ANOVA; (B) pGL-1, -2, -3, -4, and -5 ± TNF-α, p<0.01: pGL-6, -7, -8, and pGL3-basic ± TNF-α, not significant; (C) pGL-1, -2, -3, -4, -5, -6, -7 ± E₂, p<0.01: pGL-8, and pGL3-basic ± E₂, not significant; (D) pGL-1, -2, -3, -4, and -5 ± AGE-BSA, p<0.01: pGL-6, -7, -8, and pGL3-basic ± AGE-BSA, not significant; (E) there were no significant differences in relative luciferase activities among cells transfected with pGL-1, pGL-e1-11 and pGL-3’fl, which received or did not receive TNF-α, E₂ or AGE-BSA, while p<0.01 was noted between control vs. each treatment within each construct.

**FIG. 6.** Effects of the mutated NF-κB or Sp-1 sites in the inducibility of the RAGE promoter. (A) Schematic representation of mutated RAGE promoter-luciferase chimeras. (B) Relative luciferase activities in ECV304 cells transiently transfected with pGL-5 or pGL-5 NF-κB#2m in the absence (*open columns*) or presence of TNF-α (100 ng/ml), or AGE-BSA (50 µg/ml) (*closed columns* or *dotted columns*, respectively). Data were normalized by the activities of the internal control. Values are shown as the mean ± SD of nine independent experiments. (C) Luciferase activities in ECV304 cells transfected with pGL-7, pGL-7 Im, pGL-7 Im or pGL-7 ImIIm in the absence (*open columns*) or presence of E₂ (10 nM) (*closed columns*). Data were normalized by the internal control luciferase activities. Values are shown as the mean ± SD of nine independent experiments. Statistical analysis was performed using ANOVA; (B) pGL-5; control vs. TNF-α or AGE-BSA, p<0.01:
FIG. 7. Electrophoretic mobility shift (A-C) and supershift (D-F) assays of the NF-κB and Sp-1 binding sites in the RAGE promoter region. (A-C) Five µg of nuclear extracts from ECV304 cells treated for 4 h with TNF-α (100 ng/ml) (A), AGE-BSA (50 µg/ml) (B) or E₂ (10 nM) (C) were incubated with 25 fmol of the respective ³²P-end-labeled double-stranded oligodeoxyribonucleotides for 30 min as described under EXPERIMENTAL PROCEDURES. For the competition assay, a 50-fold excess of unlabeled wild-type or mutant oligodeoxyribonucleotides was added to the extracts and preincubated for 15 min prior to the incubation with the ³²P-end-labeled probe. The formation of the protein/DNA complexes as analyzed by electrophoresis on a 6% polyacrylamid gel. Closed arrows indicate NF-κB/DNA and Sp-1/DNA complexes. Open and closed arrowheads indicate non-specific binding to the probe and free probe, respectively. Essentially the same results were obtained in four independent experiments. (D-F) For the supershift assay, the indicated antibodies were added to the extracts and preincubated at 4°C for 12 h prior to incubation with the ³²P-end-labeled probe. p65, anti-human p65 antibody; p50, anti-human p50 antibody; Sp-1, anti-human Sp-1 antibody; ERα, anti-human estrogen receptor α; ERβ, anti-human estrogen receptor β. The final concentration of each antibody employed was 0.5 µg/ml. Closed arrows indicate supershifted NF-κB/DNA and Sp-1/ERα/DNA complexes. Closed arrowheads on the left indicate original NF-κB/DNA and Sp-1/ERα/DNA complexes. Open arrowheads, non-specific binding. Essentially the same results were obtained in three independent experiments.

FIG. 8. Transcriptional activation in HMVEC. (A) Relative luciferase activities in HMVEC transfected with pGL-5, pGL-5 NF-κB#2m or pGL-6 in the absence (open columns) or presence of TNF-α (100 ng/ml) (closed columns) or AGE-BSA (50 µg/ml) (dotted columns). Data were normalized by the activities of pRL-CMV-derived luciferase
used as an internal control. Values are shown as the mean ± SD of nine independent experiments. (B) Luciferase activities in HMVEC transfected with pGL-7, pGL-7 ImIIm or pGL-8 in the absence (open columns) or presence of E$_2$ (10 nM) (closed columns). Data were normalized by the activities of the internal control. Values are shown as the mean ± SD of nine independent experiments. Statistical analysis was performed using ANOVA; (A) pGL-5; control vs. TNF-α or AGE-BSA, p<0.01: pGL-5 NF-κB#2m; control vs. TNF-α, AGE-BSA, not significant: pGL-6; control vs. TNF-α, AGE-BSA, not significant; (B) pGL-7; control vs. E$_2$, p<0.01: pGL-7 ImIIm; control vs. E$_2$, not significant: pGL-8; control vs. E$_2$, not significant.
**TABLE I**

Primers used for the construction of RAGE promoter-luciferase fusion genes

| Names of the constructs | Nucleotide sequences | Corresponding nucleotide positions in the RAGE gene (33) |
|-------------------------|----------------------|---------------------------------------------------------|
|                         |                      |                                                         |
| **Forward primers**     |                      |                                                         |
| pGL-1                   | 5’-GAACGCGT AGAGATGCCAAAAATGGGGA | -1689 to -1669                                          |
| pGL-2                   | 5’-GAACGCGT CTTACCTCATACGCAGCTCA | -1599 to -1579                                          |
| pGL-3                   | 5’-GAACGCGT TACCTCGGAGGGAGTTTCTG | -1528 to -1508                                          |
| pGL-4                   | 5’-GAACGCGT GGGAAATTATTGACTGGGGCTT | -1500 to -1480                                          |
| pGL-5                   | 5’-GAACGCGT ACTGTCCCATCCTCAGCCCT | -751 to -731                                            |
| pGL-6                   | 5’-GAACGCGT TTGGAAGTGTGATGGGTGGG | -629 to -609                                            |
| pGL-7                   | 5’-GAACGCGT GAGACCCTAGAGGGTACACT | -239 to -219                                            |
| pGL-8                   | 5’-GAACGCGT CGACTGAAAGATGGGGCCTG | -89 to -69                                              |
| **Reverse primer**      |                      |                                                         |
|                         | 5’-GAAGATCT CAGGCTCCAACCTGCTGTCC | +43 to +23                                              |
| **Forward primers**     |                      |                                                         |
| pGL-e1-11               | 5’-GAGTCGAC TCCTCAGTCTGTGGGGTGAG | +52 to +72                                              |
| pGL-3’fl                | 5’-GAGTCGAC CCCACAATGATGATTAAC | +3253 to +3273                                          |
| **Reverse primers**     |                      |                                                         |
| pGL-e1-11               | 5’-GAGTCGAC AGAGCAAGATGTGTCAGGTG | +3272 to +3252                                          |
| pGL-3’fl                | 5’-GAGTCGAC AAGAGGTGGAAACAGAGAAG | +6318 to +6298                                          |
A

**TNF-α**

![Graph showing TNF-α levels with RAGE and β-actin](image)

**17β-estradiol**

![Graph showing 17β-estradiol levels with RAGE and β-actin](image)

**AGE-BSA**

![Graph showing AGE-BSA levels with RAGE and β-actin](image)
C

RAGE
β-actin

Control
TNF-α
TGF-β1
IFN-γ
17β-estradiol
4-OH tamoxifen
17β-estradiol + 4-OH tamoxifen
AGE-BSA
Non-glycated BSA

* * *

* *
A

AGE-BSA
0 10 50 100 µg/ml

VEGF$_{121}$
VEGF$_{165}$
β-actin

B

Control TNF-α 17β-estradiol AGE-BSA

RAGE
β-actin

$^*$ $^{**}$
A

RAGE

B

RAGE

rRAGE Control TNF-α 17β-estradiol AGE-BSA

RAGE

rRAGE Control TNF-α 17β-estradiol AGE-BSA
A

Control

TNF-α

RAGE

β-actin

$t_{1/2} = 2.2$

$t_{1/2} = 2.1$

17β-estradiol

AGE-BSA

RAGE

β-actin

$t_{1/2} = 2.7$

$t_{1/2} = 2.8$
B

Control

TNF-α

RAGE β-actin

RAGE β-actin

17β-estradiol

AGE-BSA

\[ t_{1/2} = 2.1 \]

\[ t_{1/2} = 2.4 \]

\[ t_{1/2} = 2.5 \]

\[ t_{1/2} = 2.8 \]
A

-1689
pGL-1

-1599
pGL-2

-1528
pGL-3

-1500
pGL-4

-751
pGL-5

-629
pGL-6

-239
pGL-7

-89
pGL-8

-1689
pGL-e1-11

-1689
pGL-3抋

-1689
pGL-1 (-1628)
AP-1 (-1542)
NF-κB (-1519)
ETS-1 (-1183)
NF-κB (-671)
NF-κB (-467)
Sp-1 (-189)
Sp-1 (-172)
Sp-1 (-45)

+43
Luc.

-1689
pGL-2

+52
3272

-1689
pGL-3抋

+3253
6318

-1599
pGL-3抋

-1528
pGL-3抋

-1500
pGL-3抋

-751
pGL-3抋

-629
pGL-3抋

-239
pGL-3抋

-89
pGL-3抋

+52
3272

-1689
pGL-3抋

+3253
6318

-1689
pGL-e1-11

+43
Luc.

-1689
pGL-3抋

+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.

-1689
pGL-3抋

+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.

-1689
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+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.

-1689
pGL-3抋

+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.

-1689
pGL-3抋

+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.

-1689
pGL-3抋

+52
3272

-1689
pGL-e1-11

+3253
6318

-1689
pGL-3抋

+43
Luc.
E

```
Control
TNF-α
17β-estradiol
AGE-BSA
```

![Graph showing the expression levels of pGL-1, pGL-e1-11, and pGL-3'fl under different conditions: Control, TNF-α, 17β-estradiol, and AGE-BSA. The y-axis represents the expression levels, and the x-axis represents the different promoters.]
|       | TNF-α | AGE-BSA | 17β-estradiol |
|-------|-------|---------|---------------|
| Wild-type excess | − | − | − |
| Mutant excess | − | − | − |

1 2 3 4

Δ

Δ

Δ

Δ

Δ

Δ

Δ

Δ

Δ
|   | D  | E  | F  |
|---|----|----|----|
|   | TNF-α | AGE-BSA | 17β-estradiol |
| p65 | + + + | + + + | + + + + |
| p50 | - + - | - + - | - + - - |
|    | 1 2 3 | 1 2 3 | 1 2 3 4 |

**Legend:**
- **TNF-α**
- **AGE-BSA**
- **17β-estradiol**
- **Sp-1**
- **ERα**
- **ERβ**

**Note:**
- The images show gel patterns with arrows indicating specific bands.
The Receptor for Advanced Glycation Endproducts Is Induced by the Glycation Products Themselves and TNF-α through NF-κB, and by 17β-estradiol through Sp-1 in Human Vascular Endothelial Cells

Nobushige Tanaka, Hideto Yonekura, Sho-ichi Yamagishi, Hideki Fujimori, Yasuhiko Yamamoto and Hiroshi Yamamoto

J. Biol. Chem. published online May 26, 2000

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