Regulation of Vascular Endothelial Growth Factor Expression by Advanced Glycation End Products*

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Advanced glycation end products (AGEs) are generated during long-term diabetes and are correlated with the development of diabetic complications, such as retinopathy. Diabetic retinopathy is characterized by an increased retinal neovascularization due to the action of the angiogenic factor, vascular endothelial growth factor (VEGF). In this report, we show that injection of insulin and glycated albumin (Alb-AGE) to mice increases VEGF mRNA expression in eyes. Insulin and Alb-AGE stimulate VEGF mRNA and protein expression in retinal epithelial cells (ARPE-19). Alb-AGE-induced VEGF expression is not modulated by the use of antioxidants, N-acetyl-L-cysteine or pyrrolidinedithiocarbamate, or by an inhibitor of phosphatidylinositol 3-kinase (PI3K), wortmannin. However, using an inhibitor of ERK activation, U0126, we show that Alb-AGE stimulates VEGF expression through an ERK-dependent pathway. Accordingly, we found that Alb-AGE activated mitogen-activated protein kinase, ERK1/2, JNK1/2, but not p38, and that Alb-AGE did not activate PI3K and PKB. Moreover, Alb-AGE activated the transcription factor, hypoxia inducible factor-1 (HIF-1) DNA binding activity. This activation is mediated by an increase in accumulation of the HIF-1α protein through an ERK-dependent pathway. Thus, stimulation of VEGF expression by Alb-AGE, through the activation of HIF-1, could play an important role in the development of diabetic retinopathy.

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Diabetes mellitus, affecting 135 million people worldwide, is characterized by a chronic state of hyperglycemia (1). Chronic hyperglycemia, per se, has several effects on cell homeostasis. Among them, hyperglycemia induces the production of advanced glycation end products (AGEs), generated by non-enzymatic reactions between glucose and free amino reactive group of proteins and lipids (2). AGEs and their intermediates have been implicated in pathophysiological dysfunction associated with the vascular complications of diabetes mellitus, such as retinopathy (3).

Diabetic retinopathy remains a leading cause of blindness in the Western world. Retinopathy is characterized by a progressive alteration of retinal microvasculature, increased vasopermeability, and the pathologic intraocular neovascularization leading to loss of vision (4). It has been proposed that an angiogenic factor released from the retina may stimulate this neovascularization. Vascular endothelial growth factor (VEGF) is such an angiogenic factor and has been linked to the development of diabetic retinopathy. Indeed, it has been observed that intraocular VEGF levels are increased in diabetic patients and that a correlation exists between the levels of glycated proteins and the development of retinopathy (5–7). Alternative splicing of VEGF mRNA leads to the formation of five distinct isoforms of 121, 145, 165, 189, and 206 amino acids (8). VEGF165 is the most predominant form of VEGF. VEGF expression is mainly regulated by tissue oxygen content (9–11) but also by growth factors and cytokines, including platelet-derived growth factor, epithelial growth factor, insulin, insulin-like growth factor-I, tumor necrosis factor α, and transforming growth factor β (12–17). Hypoxia stimulates VEGF expression, through an increase in gene transcription, a regulation at a translational level, and stabilization of the mRNA (18–20). Hypoxia regulates VEGF gene transcription by activating the transcription factor, hypoxia inducible factor-1 (HIF-1) (21, 22). HIF-1 is a basic-helix-loop-helix transcription factor, which is composed of two subunits, HIF-1α and HIF-1β. HIF-1β, also known as the arylhydrocarbon nuclear translocator, is constitutively expressed, whereas HIF-1α expression is increased upon hypoxia. In the absence of adequate signals (hypoxia or growth factor stimulation), HIF-1α is rapidly ubiquitinated by the von Hippel-Lindau tumor suppressor E3 ligase complex, and subjected to proteasomal degradation (23). Under hypoxic conditions or after stimulation with growth factors, HIF-1α is not degraded and accumulates to form an active complex with HIF-1β. Mechanisms by which cells respond to hypoxia and activate HIF-1 are not fully understood (21). It has been shown that ERK activates HIF-1 by promoting the phosphorylation of HIF-1α (24, 25) and that PI3K-dependent pathways are involved in HIF-1 activation and VEGF expression (26–28).

AGEs stimulate VEGF expression in epithelial cells and in vascular smooth muscle cells (29, 30). In vivo elimination of AGEs from the circulation occurs by macrophage scavenger receptor-mediated endocytosis in liver endothelial and Kupffer sulfonil fluoride; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidinedithiocarbamate; RAGE, receptor for advanced glycation end products.
cells (31, 32). However, during diabetes the increased concentration of AGEs result in their binding to AGE-binding proteins. Several AGE-binding proteins have been identified, including p60 homologous to OST-48, p90 homologous to 80K-H (a protein kinase C substrate), galectin-3, and RAGE (33–35). However, the signaling pathways mediated by the activation of these AGE-binding proteins are poorly understood. Cell treatment with glycated proteins leads to generation of reactive oxygen species, activation of ERK1/2, and activation of the transcription factor NF-kB (36, 37). The signaling pathways activated by AGEs and implicated in VEGF expression remain to be defined. In this report, we found that AGEs stimulate VEGF mRNA expression through an increase in HIF-1α accumulation and activation of HIF-1. Moreover, AGEs-induced VEGF expression and HIF-1α accumulation are dependent on ERK.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human glycated albumin was purchased from Sigma Chemical Co. (St. Louis, MO). Each lot was tested for insulin-like growth factor-1 contamination by IGF-1-D-RIA-CT (BIOSOURCE Europe, Nivelles, Belgium). cDNA for VEGF 165 was obtained from J. Plouet, Institut de Pharmacologie et de Biologie Structurale, Toulouse, France). Antibody to ERK1/2 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibody to the dually phosphorylated ERK was purchased from Promega Inc. (Madison, WI). Antibody to PK3 was a gift from B. Hemmings (F. Miescher Inst., Basel, Switzerland). Antibodies to phospho-PKB (Ser-473), to phosphorlylated SAPK/JNK, to p38 MAPK and to phosphorylated p38 MAPK were purchased from New England Biolabs (Beverly, MA). Antibodies to JNK1/2 and to VEGF were purchased from PharMingen (San Diego, CA). Antibody to HIF-1α was purchased from Novus Biologicals, Inc. (Littleton, CO). Antibody to CREB was a gift from S. Tartare-Deckert (INSERM 145, Nice, France) and M. Montminy (Salk Institute for Biological Studies, La Jolla, CA).

All chemical reagents were purchased from Sigma (St. Louis, MO). U0126 was purchased from Promega Inc. (Madison, WI). Culture media were purchased from Life Technologies, Inc. (Gaithersburg, MD).

**Animals and Experimental Design**—Male OF1 mice (20–22 weeks old) (Iffa-Credo, L’Arbresle, France) were deprived of food for 24 h prior to intraperitoneal injection with insulin (Actrapid Hinge, Novo-Nordisc, Denmark), glycated albumin, or saline. Animals were euthanized by cervical dislocation, and the eyes were removed, weighed, and processed for preparation of total RNA.

**Cell Culture**—ARPE-19 cells (Arising Retinal Pigment Epithelia cell line; ATCC no. CRL-2302) were grown in F12/Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum (Life Technologies, Inc.) at 37 °C with 5% CO2. 

**RNA Isolation and Northern Blot Analysis**—TRizol reagent (Life Technologies, Inc.) was used to extract total cellular RNA from tissues or confluent cells grown in 100-mm tissue culture plates according to the manufacturer’s instructions. Cells were serum-deprived overnight in medium containing 0.2% (v/v) bovine serum albumin and cells were pretreated or not with inhibitors for 30 min and stimulated for indicated times. RNA was extracted, and 10 μg of total RNA was denatured in formamide and formaldehyde and separated by electrophoresis in formaldehyde-containing agarose gels. RNA was transferred to Hybond-N membranes (Amer sham Biosciences AB, Uppsala, Sweden) and cross-linked to the membrane by UV radiation. Probes were labeled with [γ-32P]dATP by random priming using the Rediprime kit (Amer sham Biosciences, Inc.) and purified with the Probequant kit (Amer sham Biosciences, Inc.). Hybridizations were performed at 42 °C in NorthernMax hybridization buffer (Ambion, Inc., Austin, TX). Membranes were washed in 1× SSC, 0.5% SDS, and radioactivity was quantitated using a Storm 840 PhosphorImager, Molecular Dynamics.

**Western Blot**—Serum-starved cells were treated with ligands, chilled to 4 °C, and washed with ice-cold phosphate buffered saline (PBS: 140 mM NaCl, 5 mM KCl, 6 mM Na2HPO4, 1 mM KH2PO4, pH 7.4), and solubilized with lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 100 mM NaF, 2 mM vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4, 1% Triton X-100) for 20 min at 4 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred by electroblotting to nitrocellulose membranes (Hybond C, Amersham Biosciences, Inc.). The membranes were soaked first in blocking buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% (v/v) bovine serum albumin or nonfat milk and then soaked in blocking buffer containing antibodies. After washes, proteins were detected using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences, Inc.).

**Nuclear Extract Preparation**—Nuclear extracts were prepared as previously described (38). Serum-starved cells were treated with ligands, chilled to 4 °C, and washed with ice-cold PBS. Cells were scraped into 5 ml of PBS and pelleted by centrifugation at 1500 rpm for 10 min at 4 °C. Cell pellets were washed with four packed cell volumes of buffer A (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, supplemented with 2 mM DTT, 0.4 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 mM Na3VO4). Nuclear proteins were extracted by stirring at 4 °C for 30 min. After centrifugation at 13,500 rpm for 30 min, the supernatant was dialyzed against buffer B-100 (25 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, 20% (v/v) glycerol, 1.5 mM MgCl2) supplemented with 2 mM DTT, 0.4 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 mM Na3VO4. Nuclear proteins were extracted by stirring at 4 °C for 30 min. After centrifugation at 13,500 rpm for 30 min at 4 °C and designated as crude nuclear extract. The nuclear extracts were aliquoted, frozen in liquid N2, and stored at −80 °C. Protein concentration was determined by a Bio-Rad assay using bovine serum albumin as standard.

**Electrophoretic Mobility Shift Assay**—Sense and antisense oligonucleotides corresponding to the erythropoietin promoter 5′-GATCCG-CCTACCTGCTGTCCTCA-3′ were used (39). Oligonucleotides were annealed, and the double-stranded oligonucleotide (10 pmol) was labeled with T4 polynucleotide kinase and [γ-32P]dATP. The probe was purified with the Probequant kit (Amer sham Biosciences, Inc.).

Binding reactions were performed as described previously (38). Reactions contained 10 μg of nuclear extract and 0.1 μg of denatured calf thymus DNA (Sigma) in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, and 5% (v/v) glycerol. After preincubation for 5 min at room temperature, probe (2.5 × 105 cpm) was added and the incubation was continued for an additional 15 min, after which the reaction mixtures were loaded onto 5% non-denaturing polyacrylamide gel. Electrophoresis was performed at 185 V in 0.25× TBE (22.5 mM Tris-HCl, 22.5 mM boric acid, and 1.25 mM EDTA) at 4 °C. Gels were vacuum-dried, and radioactivity was determined on a Storm 840. Competitor DNAs were preincubated with nuclear extract and salmon sperm DNA for 5 min prior to addition of labeled probe.

**RESULTS**

**Glycated Albumin Increases VEGF mRNA Expression**—To study the regulation of VEGF mRNA expression, mice were injected with saline solution alone (control) or with insulin, albumin (Alb), or glycated albumin (Alb-AGE). After the indicated times, eyes were removed from the animals, and RNA was extracted and analyzed by Northern blotting using a VEGF165 cDNA probe. RNA loading and integrity were verified by a Northern blot using 18 S as a probe, and expression of VEGF mRNA was normalized as the ratio of VEGF mRNA over 18 S (Fig. 1).

We observed that both insulin and Alb-AGE induced a 2-fold increase in the level of VEGF mRNA in eyes of mice compared with control injection. Albumin alone had no effect on VEGF expression. Because Lu et al. (29) have shown that in retina the VEGF mRNA level is increased in the ganglion, inner nuclear layer, the retinal pigment epithelial, and the choroids, we have chosen here to study the effect of AGEs on VEGF mRNA expression in retinal epithelial cells.

**Glycated Albumin Stimulates VEGF mRNA and Protein Expression in Retinal Epithelial Cells**—Human retinal epithelial cells (ARPE-19) were treated for 6 h with Alb-AGE, insulin, or with both molecules, and RNA was extracted. RNA was analyzed by Northern blotting for VEGF mRNA expression (Fig. 2A). As observed, Alb-AGE and insulin induced a 2- and 3-fold...
increase in VEGF mRNA expression, respectively. Co-treatment with Alb-AGE and insulin had an additive effect, because together they induced a 5-fold increase in VEGF mRNA expression.

As shown in Fig. 2B, we verified that the increase in VEGF mRNA expression was associated to an increase in the protein levels. ARPE-19 cells were treated with Alb-AGE or with insulin, and whole cell lysates were analyzed by Western blotting using antibody to VEGF. Both Alb-AGE and insulin stimulated the expression of VEGF isoforms (VEGF121, VEGF165, VEGF189) in ARPE-19 cells. Signaling pathways involved in insulin-induced VEGF expression have been studied (17). In contrast, the regulation of VEGF mRNA expression in response to glycated proteins is poorly documented. For this reason, we have focused our work on the signaling pathways activated by AGEs and implicated in VEGF expression.

**Signaling Pathways Implicated in Alb-AGE-induced VEGF mRNA Expression**—Because stimulation of cells with Alb-AGE has been shown to produce reactive oxygen species (36), we investigated whether antioxidants modulate Alb-AGE-stimulated VEGF expression. ARPE-19 cells were treated with Alb-AGE in the absence or presence of antioxidants, N-acetyl-L-cysteine (NAC) or pyrrolidinedithiocarbamate (PDTC). RNA was extracted and analyzed by Northern blotting using a VEGF165 cDNA probe. As shown in Fig. 3A, we observed that the level of VEGF mRNA detected after Alb-AGE treatment is increased by the use of NAC and is not modified by PDTC treatment. The observation that NAC increases VEGF mRNA level could be due to its ability to activate HIF-1 (40).

Then, we investigated the role of ERK and PI3K on Alb-AGE-induced VEGF mRNA expression. ARPE-19 cells were treated with Alb-AGE in the absence or presence of specific inhibitors for MEK, U0126, or for PI3K, wortmannin. RNA was extracted and Northern blot analysis was performed using a VEGF165 cDNA probe (Fig. 3B). Alb-AGE stimulated VEGF mRNA expression in ARPE-19 cells (5-fold increase). Inhibition of MAPK activation by U0126 blocked the Alb-AGE-induced VEGF mRNA expression, whereas inhibition of PI3K by wortmannin did not seem to affect the ability of Alb-AGE to induce VEGF mRNA expression.

In conclusion, our data show that Alb-AGE stimulates VEGF expression through an ERK-dependent pathway.

**Glycated Albumin Activates MAPKs but Not PI3K/PKB**—We investigated the signaling pathways activated by Alb-AGE. First, we studied the ability of Alb-AGE to activate the MAPK family members, extracellular signal-related kinases (ERK), Jun amino-terminal kinases (JNK), and p38 MAPK. ARPE-19 cells were stimulated with Alb-AGE for 10 or 20 min, and whole cell lysates were analyzed by Western blotting using antibodies to phosphorylated forms of JNK, ERK, or p38 MAPK (Fig. 4, A–C). Alb-AGE activated JNK1 and JNK2. Maximal activation to phosphorylated forms of JNK, ERK, or p38 MAPK (Fig. 4, A–C). Alb-AGE activated JNK1 and JNK2. Maximal activation of ERK1 and ERK2 by Alb-AGE was reached within 10 min of stimulation. However, we were unable to detect any activation of p38 MAPK after Alb-AGE treatment of ARPE-19 cells.

To determine whether Alb-AGE activates the PI3K-dependent cascade, we measured the phosphorylation of a downstream effector of PI3K, PKB (Fig. 4D). ARPE-19 cells were treated with Alb-AGE or insulin, and whole cell lysates were analyzed by Western blotting using antibodies to PKB phosphorylated on serine residue 473 or to total PKB. We observed that insulin induced a rapid and strong phosphorylation and activation of PKB, whereas no phosphorylation of PKB could be
detected after Alb-AGE treatment. Using a PI3K assay, we did not detect activation of PI3K in response to glycated albumin (data not shown).

**Glycated Albumin Stimulates HIF-1α Accumulation**—To study whether glycated proteins stimulate VEGF expression through HIF-1 activation, we first investigated whether Alb-AGE induced an accumulation of HIF-1α. ARPE-19 cells were treated with Alb-AGE or with a known inducer of HIF-1α, CoCl2, for 4 h, and cell lysates or nuclear extracts were analyzed by Western blotting using an antibody to HIF-1α (Fig. 5, A and B). Expression of HIF-1α was normalized using a Western blot with antibodies to Shc or to CREB (cAMP-response element-binding protein). Expression of the HIF-1α protein was increased upon Alb-AGE stimulation (2.5-fold) in both total cell lysates and in the nucleus. As expected, CoCl2 treatment of cells led to a greater accumulation of HIF-1α in ARPE-19 cells (10-fold stimulation).

**Glycated Albumin Stimulates HIF-1 DNA Binding Activity**—Because Alb-AGE was found to stimulate HIF-1α accumulation, we determined whether this accumulation was correlated with an activation of HIF-1 DNA binding activity. ARPE-19 cells were treated in the absence or presence of Alb-AGE, and nuclear extracts were isolated. Double-strand oligonucleotides containing the hypoxia response element site present in erythropoietin promoter were used to measure the ability of HIF-1 to bind to a specific sequence (Fig. 5C). We observed that Alb-AGE induced an increase in the DNA binding activity of HIF-1α. This binding is specific, because it is abolished in the presence of an excess of unlabeled oligonucleotides.

**Accumulation of HIF-1α in Response to Alb-AGE Is Dependent on ERK**—Because Alb-AGE stimulated VEGF mRNA expression through an ERK-dependent pathway (Fig. 3), we determined whether ERKs are implicated in the induction of HIF-1α in response to AGES. ARPE-19 cells were pretreated with wortmannin or U0126 and then stimulated with Alb-AGE. Whole cell lysates were analyzed by Western blotting using antibodies to HIF-1α or to Shc (Fig. 6). As a positive control, we used CoCl2 to stimulate HIF-1α accumulation. Alb-AGE induced a 3-fold increase in HIF-1α expression, which was not affected by pretreatment with the PI3K inhibitor, wortmannin. Inhibition of ERK activation by U0126 completely inhibited the expression of HIF-1α in response to Alb-AGE. In conclusion, we found that Alb-AGE stimulated HIF-1α expression through an ERK-dependent pathway.

**DISCUSSION**

In the present study, we showed that Alb-AGE stimulates VEGF mRNA expression in an ERK-dependent pathway.
found that AGEs activate ERK and JNK but not p38 MAPK. Activation of ERK has been observed in numerous cell types (36, 37). However, activation of stress-activated protein kinases (SAPK), JNK and p38, is more controversial. In C6 glioma cells, activation of RAGE, a receptor for AGEs, leads to activation of both JNK and p38 MAPK (41). In contrast, in THP-1 monocytes, AGEs activate only p38 MAPK, without activation of JNK (42). These observations suggest that activation of MAPKs could be cell-specific. However, SAPK are not involved in the activation of the transcription of VEGF mRNA but in its stability (43).

We did not detect activation of PI3K/PKB by AGEs. Such AGEs action has been reported only in Jurkat and in PC12 cells (44). Because AGEs activate neither PI3K nor PKB in ARPE-19 cells, it is possible that PI3K activation is also cell-specific.

AGEs stimulate VEGF expression through the accumulation of HIF-1α and the subsequent activation of the transcription factor HIF-1. The induction of HIF-1α by AGEs occurs through an ERK-dependent pathway. This is in agreement with results from Agani and Semenza (45), who show that ERKs are involved in HIF-1α accumulation in response to mersalyl, an organomercurial compound. ERKs have also been implicated in VEGF expression in response to hypoxia by phosphorylating HIF-1α subunit and then leading to an activation of the transcriptional activity of HIF-1 (24, 25).

However, HIF-1 expression and activity can be regulated through other mechanisms. Indeed, overexpression of an activated form of PI3K or PKB, or expression of dominant-negative phosphatase and tensin homolog stimulates HIF-1α expression in response to hypoxia and induces angiogenesis (26, 27). Moreover, epidermal growth factor regulates HIF-1α expression through the signaling cascade PI3K/PKB/FRAP (FKBP-rapamycin-associated protein) (28). Additional studies are required to determine whether PI3K- or ERK-dependent signaling pathways modulate the degradation of HIF-1α through the proteasome.

Finally, we have observed that, compared with insulin or glycated albumin alone, a cotreatment of ARPE-19 cells with both insulin and glycated albumin has a synergistic action on expression of VEGF mRNA. Insulin induces VEGF expression mainly through a PI3K-dependent pathway (17). Because AGEs stimulate VEGF expression through an ERK-dependent pathway, it is possible that the two polypeptides use distinct signaling pathways, leading to an additive action on VEGF expression.
Regulation of VEGF by AGEs

In conclusion, to the best of our knowledge, this is the first report showing that AGEs stimulate VEGF expression through an ERK-dependent pathway. Moreover, our results suggest that AGEs-induced expression of VEGF is dependent on the transcription factor HIF-1. Based on these observations, we suggest that blockage of HIF-1 activity by ERK inhibitors could be used as a therapeutic approach to inhibit AGE-induced neovascularization during diabetes.

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