Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE

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Wautier, Marie-Paule, Olivier Chappey, Stefano Corda, David M. Stern, Ann Marie Schmidt, and Jean-Luc Wautier. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab 280: E685–E694, 2001.—Engagement of the receptor for advanced glycation end products (RAGE) by products of nonenzymatic glycation/oxidation triggers the generation of reactive oxygen species (ROS), thereby altering gene expression. Because dissection of the precise events by which ROS are generated via RAGE is relevant to the pathogenesis of complications in AGE-related disorders, such as diabetes and renal failure, we tested the hypothesis that activation of NADPH oxidase contributed, at least in part, to enhancing oxidant stress via RAGE. Here we show that incubation of human endothelial cells with AGEs on the surface of diabetic red blood cells, or specific AGEs, (carboxymethyl)lysine (CML)-modified adducts, prompted intracellular generation of hydrogen peroxide, cell surface expression of vascular cell adhesion molecule-1, and generation of ROS through an oxidation mechanism in a manner suppressed by treatment with diphenyliodonium, but not by inhibitors of nitric oxide. Consistent with an important role for NADPH oxidase, although macrophages derived from wild-type mice expressed enhanced levels of tissue factor upon stimulation with AGE, macrophages derived from mice deficient in a central subunit of NADPH oxidase, gp91phox, failed to display enhanced tissue factor in the presence of AGE. These findings underscore a central role of NADPH oxidase in AGE-RAGE-mediated generation of ROS and provide a mechanism for altered gene expression in AGE-related disorders.

THE RECEPTOR (R) for advanced glycation end products (AGE), or RAGE, a multiligand member of the immunoglobulin superfamily of cell surface molecules, engages distinct ligands, thereby leading to altered gene expression in a range of cell types (28, 45, 46). Interaction of the products of nonenzymatic glycation and oxidation of proteins/lipids, AGEs, with RAGE initiates a cascade of signal transduction events involving, at least in part, p21ras, p44/p42 mitogen-activated protein kinases, and NF-κB (10, 13, 21, 50, 56). Because the accumulation of AGEs has been linked to cellular perturbation in diabetes, renal failure, amyloidosis, and inflammation (1, 3, 6, 33, 48), understanding the precise mechanisms by which these modified adducts modulate cellular properties is a critical step in understanding the biology of this class of molecules. Recent studies have shown that specific AGEs, Nε(carboxymethyl)lysine (CML) adducts of proteins, the most prevalent AGEs found in vivo (14, 37), interact with RAGE to activate signal transduction pathways (16), ultimately leading to expression of proinflammatory genes. Transient transfection of a form of RAGE, lacking the intracellular domain but possessing the extracellular and transmembrane components, into endothelial cells (EC) or murine BV2 macrophages, preserved the ability to bind ligand but imparted a “dominant negative” (DN) effect upon cellular ligation of CML adducts. Specifically, CML-mediated activation of NF-κB by CML-ovalbumin was markedly suppressed in DN-RAGE-transfected cells vs. mock-transfected cultured cells bearing vector alone, thus supporting the concept that RAGE is a signal transduction receptor for AGEs such as CML adducts (16).

A key consequence of the interaction of AGEs, either those prepared in vitro (such as AGE- or CML-modified adducts of proteins) or those formed endogenously in vivo (such as AGE-β2-microglobulin), AGEs formed on the surface of diabetic red blood cells, or AGEs immunosolated from the serum of patients with diabetes or renal failure, with RAGE is the generation of reactive oxygen intermediates (ROI<sub>s</sub>) (27, 44, 45, 51, 52, 56). In vitro and/or in vivo, AGE-RAGE interaction resulted in generation of thiobarbituric acid reactive substances (TBARS), increased mRNA for heme oxygenase-1, enhanced nuclear translocation of NF-κB, and increased endothelial expression of vascular cell adhesion mole-

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E685
cule-1 (VCAM-1) and endothelial permeability (44, 51, 52, 56). In each of these cases, the effects of AGE were mediated by RAGE, as indicated by suppression in the presence of anti-RAGE IgG, or by soluble (s)RAGE, the extracellular ligand-binding domain of the receptor. In addition, these studies suggested that generation of ROI and enhanced oxidant stress was a potent factor initiating signal transduction and altered gene expression, as AGE-RAGE-mediated effects were inhibited in the presence of antioxidants such as N-acetylcysteine (NAC), probucol, or vitamin E. Consistent with this concept, in vascular smooth muscle cells, AGE-RAGE-mediated activation of ERK 1/2 kinases was enhanced in the presence of glutathione depletion (21).

Previously, Lander and colleagues (19, 20) demonstrated that ROIs generated in the cellular milieu directly activated p21ras; in those experiments the cysteine at position 118 was a critical residue responsive to ROI. Consistent with the concept that AGE-RAGE-mediated generation of ROIs was the key stimulus leading to activation of signal transduction pathways, RAGE-bearing PC12 cells stably transfected to express mutant p21ras, in which Cys118 was mutated to a serine, displayed complete suppression of activation of ERK 1/2 kinases upon exposure to AGE-albumin. In contrast, AGE-mediated activation of ERK 1/2 kinases in PC12 cells overexpressing wild-type p21ras was intact (21).

Thus a key challenge raised by these observations was to identify the precise molecular mechanism(s) by which ROIs were generated consequent to cellular ligation of RAGE by AGE. Here, we tested the hypothesis that activation of NADPH oxidase by AGE-RAGE interaction contributed, at least in part, to the generation of ROIs and initiation of a cascade of signal transduction events leading to altered gene expression in the cellular microenvironment.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC) were prepared and characterized from human umbilical cords as previously described (52, 53). Purity of HUVEC cultures was assessed as previously described (53). Murine peritoneal macrophages were obtained after intraperitoneal administration of Brewer’s thioglycollate broth (4%) (Difco, Detroit, MI) according to previously described methods (25). Four days after administration, peritoneal macrophages were harvested by flushing the peritoneal cavity with cold DMEM (Life Technologies, Grand Island, NY). The retrieved cells were washed twice with Hank’s buffered salt solution (HBSS) and then plated onto 96-well microplates (Nunc, Naperville, IL) at a density of 5 × 10^5 cells/well. After 16 h of incubation at 37°C, nonadherent cells were removed by washing with HBSS. Adherent cells were cultured in RPMI 1640 medium containing fetal bovine serum (FBS; 10%). Adherent cells were >96% macrophages as assessed by Giemsa staining and by immunostaining with anti-CD14 IgG.

Preparation of normal or diabetic red blood cells. Blood was obtained from normal subjects (n = 11) and patients with type 1 diabetes (n = 15) in accordance with the provisions of the Declaration of Helsinki and the rules of our institutions. The mean level of glycemia and %glycosylated hemoglobin observed in diabetic patients was 14.6 ± 3.3 mmol/l and 13.7 ± 1.7%, respectively. Normal volunteers were from the Blood Transfusion Center, and diabetic patients were hospitalized in the Department of Internal Medicine. Venous blood was anticoagulated in trisodium citrate (0.129 M) and tested within 24 h of collection. Normal red blood cells (NRBCs) or diabetic red blood cells (DRBCs) were washed three times with saline and two times in HBSS. Cells were resuspended in HBSS (2.5 × 10^5 cells/ml) (51). To prevent the potential generation of reactive oxygen species (ROS) by contaminating leukocytes, RBC preparations were depleted of leukocytes by use of a leukocyte removal filter (Sepacell, Asahi, Tokyo, Japan). After this procedure, leukocyte contamination was <2 leukocytes per 10^6 RBCs.

Preparation of glycated proteins. AGE-ovalbumin (AGE-OVA) was prepared under sterile conditions by incubation of OVA (10 mg/ml) in HBSS containing glucose 6-phosphate (G-6-P, 0.25 M), protease inhibitors [pepstatin A, 0.1 μg/ml; leupeptin, 0.5 μg/ml; aprotinin, 2 μg/ml; and phenylmethylsulfonyl fluoride (PMSF), 0.0015 M], EDTA (0.001 M), and glycine (0.001 M) at 37°C for 60 days in the dark. After this incubation period, the mixture was dialyzed vs. HBSS (28, 45, 46). Control OVA was prepared in an identical manner, except that G-6-P was omitted. General chemical reagents were obtained from Sigma (St. Quentin, France or St. Louis, MO) unless otherwise indicated. AGE-OVA was characterized, as previously described, by fluorescence and binding to immobilized RAGE in plastic dishes (28, 45). To prepare CML-OVA, OVA and sodium cyanoborohydride were dissolved in sodium phosphate buffer (0.2 M; pH 7.8) (16). Glyoxylic acid was then added, and the mixture was incubated for 24 h at 37°C. Control proteins were prepared under the same conditions, except that glyoxylic acid was omitted (16). Preparations of CML-modified proteins were extensively dialyzed vs. PBS and characterized by percent modification as determined both by employment of 2,4,6-trinitrobenzenesulfonic acid to determine the difference in lysine residues of modified vs. unmodified preparations (9) and by gas chromatography-mass spectroscopy (32). Different levels of modification of CML-OVA (16) were employed in these studies to more closely reflect pathophysiologically relevant degrees of CML modification (4). All CML-modified, AGE-modified, and control proteins were devoid of endotoxin before experiments by chromatography onto Detoxi-gel columns (Pierce, Arlington Heights, IL). The level of endotoxin in all protein preparations (concentration range, 2–6 mg/ml) was <3 μg/ml (Sigma).

Antibodies and soluble RAGE. Monospecific polyclonal antibodies to human RAGE were prepared and characterized as previously described (28, 45). Soluble recombinant rat RAGE was produced in a baculovirus expression system and purified to homogeneity as previously described (39).

Measurement of extracellular H2O2. Measurement of H2O2 production by HUVEC was based on the horseradish peroxidase (HRPO)-dependent conversion of phenol red by H2O2 into a compound with increased absorbance at 610 nm (34). Briefly, confluent HUVEC in plastic dishes (35 mm) were incubated in buffer containing NaCl (0.14 M), potassium phosphate (0.01 M; pH 7.0), dextrose (0.0055 M), phenol red (0.00056 M), and HRPO (19 U/ml; Sigma). HUVEC were exposed to the indicated mediators for 30 min at 37°C. Where indicated, superoxide dismutase (SOD; 300 U/ml; Sigma), catalase (100 U/ml; Sigma), probucol (50 μM; Sigma), or N-acetylcysteine (NAC; 0.03 M; Sigma) was added to the incubation buffer. At the end of the incubation period, the supernatant was assayed in a spectrophotometer at 610 nm (Ultraspec-Plus, Pharmacia, Uppsala, Sweden). At the end of
the experiment, cell number and viability were assessed by determination of the percentage of cells excluding trypan blue (Sigma). Standard curves employing H$_2$O$_2$ (0.1–50 μM) were established for each assay.

**Measurement of intracellular H$_2$O$_2$.** Conversion from a nonfluorescent to a fluorescent form of dihydrothoradamine 123 is relatively specific for detection of ROS (41). Confluent HUVEC, in 100-mm plastic dishes, were incubated with dihydrothoradamine 123 (5 μM; Molecular Probes Europe, Leiden, The Netherlands) in phenol-free medium 199 (Life Technologies) for 1 h at 37°C. HUVEC were exposed to the indicated mediators for 4 h at 37°C in the presence of dihydrothoradamine 123 (5 μM). Where indicated, mediators were co-incubated with dipherylidonium chloride (31) (DPI; 5 μM; Alexis, San Diego, CA), N’-monomethyl-l-arginine (l-NMMA; 0.001 M; Alexis), rat sRAGE (60 μg/ml), probucol (50 μM), SOD (300 U/ml), or catalase (100 U/ml). Fluorescent intensity was measured using a Farrand system 3 spectrofluorometer (Beckman, Fullerton, CA). ROS in endothelial cells were also measured using the probe 2,7-dichlorofluorescein (DCFH) by ROS, particularly hydrogen peroxide and hydroxyl radical, yields the fluorescent product DCF (40). EC monolayers on 25-mm-diameter plastic coverslips (Thermanox, Nunc) were placed in a stainless steel flow-through chamber (1 ml volume, Penn Century, Philadelphia, PA). The chamber was sealed using thin wafer gaskets and mounted on an inverted microscope. A water-jacketed glass equilibration column (Radnotti Glass Technology, Monrovia, CA) was used to equilibrate the perfusate with a 25% O$_2$-5% CO$_2$-70% N$_2$ air mixture. After 1 h of dye uptake and stabilization in Krebs-Henseleit bicarbonate buffer (pH 7.4), HUVEC were stimulated with either CML-OVA (60 μg/ml) or control OVA (60 μg/ml) diluted in Krebs-Henseleit buffer. As indicated, HUVEC were co-incubated with DPI (5 μM), l-NMMA (1 mM), 4′-hydroxy-3′-methoxycetophenone (HMAP; 100 μg/ml) (42), rat sRAGE (60 μg/ml), or anti-RAGE IgG (100 μg/ml). Flow was stopped, and observation was carried out for 1 h. Fluorescent images were acquired every 15 min and analyzed using Metamorph software (Metamorph Imaging System) (5). Fluorescence intensity values were presented as the percentage of the initial value recorded after 1 h post-loading.

**Measurement of extracellular superoxide.** Measurement of superoxide ion was based on capacity to reduce ferricytochrome c in ferrocyanochrome at pH 7.8. Confluent HUVEC, in 35-mm plastic dishes, were incubated in phenol-free medium 199 (0.5 ml; Life Technologies) containing cytochrome c (160 μM; Sigma). HUVEC were stimulated with the indicated mediators for 30 min at 37°C. At the end of the incubation period, optical density of the supernatant was measured at 550 nm. Production of superoxide (in nanomoles) was calculated as (D$_{absorbance}$ at 550 nm × 100/6.3), where D absorbance is calculated as the difference between the absorbance of reduced and oxidized forms of cytochrome c.

**Determination of VCAM-1 expression.** Confluent HUVEC in 96-well microplates were stimulated with the indicated mediators for 16 h before determination of VCAM-1 expression. Where indicated, HUVEC were incubated with mediators in the presence or absence of the indicated concentration of DPI, l-NMMA (0.001 M), HMAP (100 μg/ml), rat sRAGE (60 μg/ml), or anti-RAGE IgG (100 μg/ml). VCAM-1 expression was measured using mouse anti-human VCAM-1 IgG (R&D Systems, Abington, Oxon, UK). Binding was revealed with a second antibody [125I-labeled sheep anti-mouse IgG F(ab’)$_2$ fragment; Amersham, Les Ulis, France]. Cells were washed, solubilized with SDS (0.1%)-NaOH (0.025 M), and collected. Radioactivity was measured in a gamma counter (Beckman). Specific binding was calculated by subtracting nonspecific radioactivity measured in wells containing only 125I-labeled isotype-matched nonbinding EIA monoclonal antibody (a generous gift from Dr. M. Bevilacqua, UCDS, Howard Hughes Medical Institute, La Jolla, CA).

**Determination of tissue factor activity.** HUVEC (1.2 × 10³ cells/well) or mouse macrophages (1 × 10⁴ cells/well) were plated for 18 h in 96-well microplates previously coated with gelatin (0.2%). HUVEC were then incubated in triplicate with the indicated mediators for 6 h at 37°C. Mouse macrophages were incubated with AGE-OVA for 16 h at 37°C. Where indicated, incubation of mediators was performed with DPI (5 μM), l-NMMA (0.001 M), sRAGE (60 μg/ml), or probucol (50 μM). Procoagulant activity was assessed by an amplydoytic assay using a factor Xa-specific chromogenic substrate (CBS 31–39, Stago, Asnières, France) according to previously published methods (36). At the end of the incubation period, medium was removed, and the cells were washed three times in Tris-buffered saline (TBS) containing Tris-HCl (0.05 M; pH 8.4) and NaCl (0.2 M). HUVEC or macrophages were incubated simultaneously with normal pooled human or mouse plasma, respectively (0.05 ml), and recombinant human hirudin (4 U/ml; Stago) and CBS 31–39 (0.05 ml) chromogenic substrate were supplemented with CaCl$_2$ (0.05 M). For each plate, a standard curve from 0 to 1 mU of purified human factor Xa/well was established. At the end of the incubation, optical density was read at 405 nm using a multispec reader. Mean absorbance from four control wells (CBS 31–39 diluted in TBS supplemented with normal human plasma without cells) was subtracted from each assay. The values obtained were then fitted onto a standard curve.

**Animal studies.** Male gp91-phox null mice (backcrossed 11 generations into the C57Bl/6 background) were a generous gift from Dr. Mary Dinauer (Indiana University School of Medicine, Indianapolis, IN) (17, 35).

**Statistical analysis.** Results are expressed as means ± SE. Statistical analysis was performed by one-way ANOVA to compare results between diabetic RBCs or AGE/CML-OVA and control experimental conditions, followed by the parametric Dunnett’s test.

**RESULTS**

To test the concept that NADPH oxidase was a central target of AGE-RAGE interaction in the cellular milieu by which ROIs were generated within the cell, we studied the effects of AGEs on RAGE-bearing HUVEC and in vivo-derived murine macrophages. Our previous studies demonstrated that DRBCs bore cell surface AGEs; these AGEs engaged cell surface RAGE on HUVEC and in vivo-generated murine macrophages. To test the concept that NADPH oxidase was a central target of AGE-RAGE interaction in the cellular milieu by which ROIs were generated within the cell, we studied the effects of AGEs on RAGE-bearing HUVEC and in vivo-derived murine macrophages. Our previous studies demonstrated that DRBCs bore cell surface AGEs; these AGEs engaged cell surface RAGE on HUVEC and in vivo-generated murine macrophages. Our previous studies demonstrated that DRBCs bore cell surface AGEs; these AGEs engaged cell surface RAGE on HUVEC and in vivo-generated murine macrophages. Our previous studies demonstrated that DRBCs bore cell surface AGEs; these AGEs engaged cell surface RAGE on HUVEC and in vivo-generated murine macrophages.
Fig. 1. Incubation of human umbilical vein endothelial cells (HUVEC) with diabetic red blood cells (DRBC) or (carboxymethyl)lysine (CML)-modified adducts enhances generation of H$_2$O$_2$. A: extracellular generation of H$_2$O$_2$ by DRBC. HUVEC were incubated for 30 min at 37°C with either normal RBC (NRBC) or DRBC ($2.5 \times 10^9$). Where indicated, DRBC were incubated in the presence or absence of the soluble receptor for advanced glycation end products (sRAGE; 60 µg/ml), superoxide dismutase (SOD; 300 U/ml), catalase (100 U/ml), probucol (50 µM), or N-acetylcysteine (NAC; 0.03 M). Measurement of H$_2$O$_2$ was performed as described in MATERIALS AND METHODS. Results are means ± SE of 5 different experiments. *$P$, 0.05; **$P$, 0.01. B and C: intracellular generation of H$_2$O$_2$ by DRBC (B) or CML-modified adducts of protein (C). B: HUVEC were incubated with NRBC or DRBC for 4 h at 37°C with dihydrorhodamine 123 (5 µM) at 37°C. Where indicated, DRBC were incubated in the presence or absence of rat sRAGE (60 µg/ml), catalase (100 U/ml), SOD (300 U/ml), probucol (50 µM), diphenyliodonium chloride (DPI; 5 µM), or N$^\omega$-monomethyl-l-arginine (l-NMMA; 0.001 M). Fluorescent intensity was measured as described in text. Results are means ± SE of 5 different experiments. **$P$, 0.01. C: endothelial cell monolayers on 25-mm-diam plastic coverslips were placed in a stainless steel flow-through chamber (1 ml volume). The chamber was sealed using thin wafer gaskets and was mounted on an inverted microscope. A water-jacketed glass equilibration column was used to equilibrate the perfusate with a 25% O$_2$-5% CO$_2$-70% N$_2$ air mixture. After 1 h of dye uptake and stabilization in Krebs-Henseleit bicarbonate buffer (pH 7.4), HUVEC were stimulated with CML-ovalbumin (OVA; 45 or 90 mmol CML/mol lysine) (60 µg/ml) or control OVA (60 µg/ml) diluted in Krebs-Henseleit buffer. Where indicated, CML-OVA (90 mmol CML/mol lysine)-stimulated HUVEC were co-incubated with rat sRAGE (60 µg/ml, anti-RAGE IgG (100 µg/ml), DPI (5 µM), or l-NMMA (1 mM). Flow was stopped and observation carried out for 1 h. Fluorescent images were acquired every 15 min and analyzed. Fluorescence intensity values were presented as % of initial value recorded after 1 h postloading. Results are means ± SE of ≥3 different experiments. *$P$, 0.05; **$P$, 0.01; ***$P$, 0.001.
to RAGE was demonstrated by suppression of DRBC-mediated generation of H$_2$O$_2$ by sRAGE (Fig. 1A). Antioxidants, including catalase, SOD, probucol, and NAC, inhibited DRBC-mediated generation of ROI in the extracellular milieu (Fig. 1A). In addition to the extracellular space, it was essential to examine the biochemical consequences of AGE-RAGE interaction in the intracellular environment, because our studies suggest that generation of ROI initiates a cascade of intracellular signaling events. To detect intracellular formation of ROI, we employed dihydrorhodamine 123. Binding of DRBC to HUVEC induced an ~2.3-fold increase in rhodamine 123 generation, indicative of H$_2$O$_2$ generation, compared with NRBC (Fig. 1B). These effects of DRBC were inhibited in the presence of sRAGE and by DPI, the latter strongly suggestive of a role for NADPH oxidase in generation of intracellular ROI (Fig. 1B). However, the antioxidants catalase and SOD were without significant effect on DRBC-mediated generation of intracellular H$_2$O$_2$, consistent with their inability to penetrate living cells. Furthermore, addition of 1-NMMA did not affect the ability of DRBC to generate intracellular H$_2$O$_2$, consistent with lack of direct involvement of nitric oxide (NO) in AGE-RAGE-mediated generation of intracellular ROI (Fig. 1B).

As an additional means to assess the extent of ROI generated intracellularly upon engagement of cell surface RAGE on HUVEC by AGE-bearing DRBC, HUVEC were loaded with DCFH and exposed to CML-OVA, a specific AGE ligand of RAGE, or native OVA. Compared with native OVA, incubation of HUVEC with CML-OVA modified with either 45 or 90 mmol CML/mol lysine resulted in a significant increase in DCFH fluorescence within 1 h, indicative of intracellular generation of ROI (Fig. 1C). These effects of CML-OVA were significantly suppressed in the presence of sRAGE, anti-RAGE IgG, and DPI (Fig. 1C); however, 1-NMMA (Fig. 1C) and nonimmune IgG (not shown) were without effect.

Our previous studies suggested that incubation of HUVEC with AGE albumin, or AGEs immunosolated from the plasma of patients with diabetes, resulted in increased transcription and translation of VCAM-1 (44). To test the concept that generation of ROI was a critical step in this process, we incubated HUVEC with DRBC. Compared with incubation of the EC monolayer with NRBC, exposure of the cells to DRBC resulted in an ~1.8-fold increase in cell surface expression of VCAM-1 (Fig. 2A). That RAGE was importantly involved in these events was demonstrated by suppression of DRBC-mediated induction of VCAM-1 in the presence of sRAGE (Fig. 2A). Strongly suggestive of a role for activation of NADPH oxidase was the finding that marked suppression of DRBC-mediated expression of VCAM-1 resulted in the presence of DPI (Fig. 2A). It is important to indicate that the effects of DPI on DRBC-mediated expression of VCAM-1 were dose dependent. Compared with induction of VCAM-1 by DRBC in the absence of DPI (2.73 ± 0.32 cpm × 10$^3$), treatment with DPI (5 μM) resulted in an ~90% reduction in specific activity for VCAM-1 (0.359 ± 0.035 cpm × 10$^3$; $P < 0.001$ vs. absence of DPI). Compared with induction of VCAM-1 by DRBC in the absence of DPI, treatment with DPI (1 μM) resulted in an ~70% reduction in specific activity for VCAM-1 (0.814 ± 0.105 cpm × 10$^3$; $P < 0.01$ vs. absence of DPI). In the presence of DPI (0.5 μM), an ~50% reduction in specific activity for VCAM-1 was noted [1.355 ± 0.15 x 10$^3$ counts/min (cpm); $P < 0.01$ vs. absence of DPI]. Finally, in the presence of DPI (0.25 μM), an ~24% reduction in specific activity for VCAM-1 was noted (2.105 ± 0.32 cpm × 10$^3$; $P < 0.05$ vs. absence of DPI). In contrast, blockade of NO synthase (NOS) with 1-NMMA had no effect (Fig. 2A). Similar results were observed in the presence of CML-OVA; compared with incubation of HUVEC with native OVA, incubation of EC with CML-OVA, modified with 35, 45 or 90 mmol CML/mol lysine, resulted in a significant increase in cell surface expression of VCAM-1 (Fig. 2B). The effects of CML-OVA were significantly suppressed in the presence of sRAGE. In addition, preincubation of the monolayers with DPI and another inhibitor of NADPH oxidase, HMAP (42), resulted in suppression of the effects of CML-OVA (Fig. 2B). Importantly, incubation of EC monolayers with either sRAGE or DPI, in the absence of added stimulus (DRBC or CML-modified adducts), did not affect basal expression of VCAM-1 in these studies (data not shown).

Enhanced expression of tissue factor (TF), the central regulator of the procoagulant pathway in vivo (29), has been linked to diabetes and the effects of AGEs. Although HUVEC do not ordinarily produce significant levels of TF under basal conditions, incubation of EC with DRBC resulted in an ~3.1-fold increase in TF activity compared with exposure of the monolayer to NRBC (Fig. 3). These effects of DRBC were diminished in the presence of sRAGE. In addition, treatment of the monolayer with DPI resulted in suppression of DRBC-mediated increases in HUVEC generation of TF (Fig. 3). In contrast, blockade of NOS with 1-NMMA had no effect (Fig. 3).

To determine whether the observed effects of AGEs were due, at least in part, to activation of NADPH oxidase, we tested the concept that, in murine macrophages lacking a critical component of NADPH oxidase, gp91$^{phox}$, the effects of AGE would be abrogated. Exposure of wild-type murine macrophages to AGE-OVA resulted in an ~3.2-fold increase in TF activity compared with exposure of the monolayer to NRBC (Fig. 3). These effects of DRBC were diminished in the presence of sRAGE. In addition, treatment of the monolayer with DPI resulted in suppression of DRBC-mediated increases in HUVEC generation of TF (Fig. 3). Finally, blockade of NO synthase (NOS) with 1-NMMA had no effect (Fig. 3).

Consistent with an important role for NADPH oxidase in mediating the effects of AGE-RAGE, macrophages retrieved from gp91$^{phox}$ null mice displayed complete inhibition of AGE-induced TF activity (Fig. 4). Importantly, immunoblotting studies indicated that wild-type and gp91$^{phox}$-null macrophages expressed equivalent amounts of RAGE antigen (data not shown).
DISCUSSION

These findings highlight the contribution of a specific pathway, activation of NADPH oxidase, by which AGE-mediated generation of ROIs and triggering of signal transduction events lead to altered gene expression in EC and macrophages via RAGE. Although DPI may inhibit other flavoprotein dehydrogenases in addition to NADPH oxidase (26, 31), our observations that HMAP, another inhibitor of NADPH oxidase (42), similarly suppresses AGE-mediated generation of ROIs, and the finding that enhanced activity of TF in AGE-stimulated macrophages retrieved from gp91phox-null mice was suppressed compared with wild-type macrophages, strongly suggest important roles for NADPH oxidase in AGE-mediated processes. Importantly, recent studies indicating that endothelial cells express a gp91phox-containing NADPH oxidase (8) support our hypothesis that activation of this enzyme provides a source of ROI upon AGE engagement of RAGE in EC. In those studies by Gorlach et al. (8), it was shown that NADPH oxidase was a major source of ROI generation in the arterial wall, because its activation was associated with impaired bioavailability of endothelium-derived NO.

RAGE is a multiligand receptor of the immunoglobulin superfamily. In addition to AGEs, RAGE serves as
a cell surface receptor for amyloid β-peptide (Aβ), a cleavage product of the β-amyloid precursor protein that accumulates in Alzheimer’s disease (AD), and β-sheet fibrils (55, 57, 58). In vivo, blockade of RAGE in a murine model of systemic amyloidosis suppressed amyloid-induced nuclear translocation of NF-κB and cellular activation (58). RAGE is also a signal transduction receptor for extracellular newly identified RAGE binding protein (ENRAGEs) and related members of the S100/calgranulin family of proinflammatory cytokines (10). The S100/calgranulin family is comprised of closely related polypeptides released from activated inflammatory cells, including polymorphonuclear leukocytes, peripheral blood-derived mononuclear phagocytes, and lymphocytes (43, 60). Their hallmark is accumulation at sites of chronic inflammation, such as psoriatic skin disease (23), cystic fibrosis (2), inflammatory bowel disease (22), and rheumatoid arthritis (30). Ligation of RAGE by ENRAGEs mediated activation of EC, macrophages, and lymphocytes. In vivo, blockade of RAGE suppressed inflammation in murine models of delayed-type hypersensitivity and inflammatory bowel disease. In parallel with suppression of the inflammatory phenotype, inhibition of RAGE-S100/calgranulin interaction decreased NF-κB activation and expression of proinflammatory cytokines in tissues, suggesting that receptor blockade changed the course of the inflammatory response. Previous studies further indicated that RAGE was likely a receptor for amphoterin, a molecule linked to neurite outgrowth in developing neurons of the central and peripheral nervous system (12). These studies suggested that amphoterin-RAGE was linked to cellular migration and invasiveness. Consistent with this concept, the expression of amphoterin and RAGE is increased in murine and human tumors. Blockade of RAGE in vivo suppressed local growth and distant spread of implanted tumors, as well as the growth of tumors forming endogenously in susceptible mice. Consistent with an important role for RAGE-mediated signal transduction in these processes, blockade of RAGE/RAGE signaling on amphoterin-coated matrices suppressed activation of p44/42, p38, and stress-activated and c-Jun NH2-terminal protein kinases (50).

In settings characterized by increased accumulation and expression of RAGE and its ligands, such as diabetic atherosclerotic lesions and periodontal, chronic disorders such as rheumatoid arthritis and inflammatory bowel disease, and AD brain, enhanced inflammatory responses have been linked to ongoing cellular perturbation and eventual tissue/organ failure. In this context, one consequence of ligand-RAGE-mediated activation of MAP kinases and NF-κB is increased transcription and translation of VCAM-1. At the cell surface, endothelium stimulated by a range of mediators, such as lipoprotein lipase, tumor necrosis factor-α, AGES, and ENRAGEs, for example, displays increased adhesion of proinflammatory mononuclear cells, at least in part, via VCAM-1. Recent studies have suggested that the proinflammatory effects of VCAM-1 are not limited to cellular adhesion events, as binding of ligand to VCAM-1 in endothelial cell lines and primary cultures induced activation of endothelial NADPH oxidase, a process shown to be essential for lymphocyte migration through the stimulated cells (24). These findings suggest that activation of RAGE at the cell surface may initiate a cascade of events including activation of NADPH oxidase and a range of proinflammatory mediators such as VCAM-1. Once set into motion, RAGE-mediated events may then stimulate a chronic cycle of sustained proinflammatory gene expression.
Although it might be concluded from these observations that antioxidant therapy might provide a logical means to suppress inflammation and tissue injury in disorders characterized by accumulation of AGEs, studies in a range of clinical disorders, such as atherosclerosis, have not unequivocally supported this hypothesis (11, 38, 59). In this context, it is likely that in defined milieu, oxidant stress responses are critical to adaptive host defense mechanisms, such as in the case of invading pathogenic bacteria. Therefore, RAGE-induced activation at the cell surface might provide a targeted therapeutic approach. For example, in diabetes, although oxidant stress responses are essential to eliminate pathogenic periodontal pathogens, ongoing AGE/ENRAGE-mediated cellular activation in infected periodontium has been linked to increased generation of proinflammatory cytokines and tissue-destructive matrix metalloproteinases, processes leading to destruction of alveolar bone (18). Thus, targeting RAGE might provide a strategy to suppress maladaptive host responses while preserving the host’s ability to mount beneficial prooxidant defenses.

Finally, it is essential to consider that ligand-induced activation of RAGE results in generation of ROIs by routes other than NADPH oxidase in distinct milieu. For example, additional potential sources of RAGE-mediated ROI include activation of the mitochondrial respiratory chain, microsomal enzymes, xanthine oxidase, and arachidonic acid pathways (7, 47, 49, 54). Consistent with the concept that multiple sources of pathogenic ROI exist in the cellular microenvironment, it was not surprising that breeding of gp91phox-null mice with mice deficient in apolipoprotein E did not inhibit or suppress atherosclerosis (15). These observations highlight the likelihood that diverse sources of ROI in the vessel wall, such as peroxynitrite, may importantly contribute to vascular dysfunction. Although the present studies support the concept that activated NADPH oxidase is a central target of RAGE and that ROIs generated by this mechanism may significantly impact on cellular properties, a challenge of future work will be to determine the potential contribution of distinct sources of ROIs in the pathogenesis of cellular dysfunction triggered by activation of RAGE.

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