N\textsuperscript{\textdegree}-(Carboxymethyl)Lysine Adducts of Proteins Are Ligands for Receptor for Advanced Glycation End Products That Activate Cell Signaling Pathways and Modulate Gene Expression*

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Recent studies suggested that interruption of the interaction of advanced glycation end products (AGEs), with the signal-transducing receptor receptor for AGE (RAGE), by administration of the soluble, extracellular ligand-binding domain of RAGE, reversed vascular hyperpermeability and suppressed accelerated atherosclerosis in diabetic rodents. Since the precise molecular target of soluble RAGE in those settings was not elucidated, we tested the hypothesis that predominant specific AGEs within the tissues in disorders such as diabetes and renal failure, N\textsuperscript{\textdegree}-(carboxymethyl)lysine (CML) adducts, are ligands of RAGE. We demonstrate here that physiologically relevant CML modifications of proteins engage cellular RAGE, thereby activating key cell signaling pathways such as NF-\textkappaB and modulating gene expression. Thus, CML-RAGE interaction triggers processes intimately linked to accelerated vascular and inflammatory complications that typify disorders in which inflammation is an established component.

Receptor for AGE\textsuperscript{\textkappa} (RAGE), a member of the immunoglobulin superfamily, was first described as a cell surface interaction site for advanced glycation end products (AGEs), products of glycation and oxidation of proteins and lipids (1–2). AGEs are a heterogeneous class of compounds, whose accumulation in disorders such as diabetes, renal failure, Alzheimer’s disease, and, indeed, natural aging, albeit to a lesser degree, has suggested their potential contribution to the pathogenesis of complications that typify these conditions (3–7). Our previous studies demonstrated that both in vitro and in vivo derived heterogeneous AGEs ligate cell surface RAGE on endothelium (ECs), mononuclear phagocytes (MPs), vascular smooth muscle (VSMC), and neurons to activate cell signaling pathways such as ERK1/ERK2 kinases and NF-\textkappaB (8–9), thereby redirecting cellular function in a manner linked to expression of inflammatory and prothrombotic genes important in the pathogenesis of chronic disorders as apparently diverse as diabetic macrovascular disease and amyloidosis (10–20).

Our recent studies suggested that interruption of the interaction of AGEs with RAGE in vivo, by administration of soluble RAGE (sRAGE), the extracellular ligand-binding domain of RAGE, reversed vascular hyperpermeability and suppressed accelerated atherosclerotic lesion development and complexity in diabetic rodents (19–20). In the latter studies, analysis of plasma demonstrated evidence of an sRAGE/AGE complex; immunoprecipitation of plasma obtained from diabetic sRAGE-treated mice with anti-RAGE IgG yielded species immunoreactive with both anti-RAGE IgG or affinity purified anti-AGE IgG, suggesting that sRAGE might bind up AGEs and limit their interaction with and activation of cell surface RAGE. The beneficial effects of sRAGE were independent of alterations in other risk factors, such as hyperglycemia and hyperlipidemia, implicating a role for AGE-RAGE interaction in the development of vascular dysfunction in diabetes (20).

These past studies, however, did not elucidate the precise AGE(s) that trigger signal transduction mechanisms upon engagement of RAGE. We thus sought to test specific AGE structures for their ability to bind RAGE on the surface of cells such as ECs, MPs, and VSMCs in order to determine their role in cellular activation.

In this context, recent biochemical and immunohistochemical studies suggested that N\textsuperscript{\textdegree}-(carboxymethyl)lysine (CML) modifications of proteins are predominant AGEs that accumulate in vivo (21–24). Elevated serum levels of CML were demonstrated in patients with diabetes (24–25) and renal failure (26). Importantly, enhanced accumulation of CML was shown in vascular tissue, atherosclerotic lesions, and glomerular tissue retrieved from diabetic rodents and human subjects (25, 27–30). In these settings, CML adducts co-localized with oxidation epitopes, such as malondialdehyde and 4-hydroxynonenal. These observations are consistent with the concept that beyond processes mediating glycoxidation of proteins (31), lipid oxidation itself triggers generation of CML (32), thereby establishing a likely link between enhanced glycation observed in
CML Adducts Bind RAGE and Modulate Cellular Properties

Diabetic hyperglycemia and disturbances of lipid metabolism, common to both types 1 and 2 diabetes (33).

Furthermore, recent findings suggested that CML modifications may form directly as a consequence of activation of the myeloperoxidase-hydrogen peroxide-chloride system, thereby providing a mechanism for conversion of hydroxy amino acids into glycodehyde, a precursor in the steps leading to formation of CML (34). These findings may have direct implications for inflammatory processes that characterize certain complications of diabetes and renal failure, for example, as atherosclerosis, impaired wound healing, aggressive and inflammatory periodontal disease, and dialysis-related amyloidosis (DRA) (6, 35–36).

Consistent with these concepts, previous studies placed RAGE in the vascular and inflammatory milieu characteristic of disorders in which AGEs, such as CML, accumulate; indeed, expression of RAGE is enhanced in these settings, beyond that observed in normal adult tissues (37–41). Taken together, therefore, these considerations suggested that examination of CML adducts of proteins as potential active AGE ligands for RAGE was a logical step.

Here we report that CML-modified proteins engage cellular RAGE in vitro and in vivo to activate key cell signaling pathways such as the transcription factor NF-κB, with subsequent modulation of gene expression. Together, these findings link CML-RAGE interaction to the development of accelerated vascular and inflammatory complications that typify disorders in which inflammation is an established component.

EXPERIMENTAL PROCEDURES

Synthesis and Characterization of CML-modified Proteins—Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), or ovalbumin (OVA) (Sigma) (176 mg in each case), and sodium cyanoborohydride (28.3 mg; 0.49 μM) were dissolved in sodium phosphate buffer (0.2 M, pH 7.8); to this was added glyoxylic acid (14.3 mg; 0.155 M) in a total volume of 1 ml per reaction. The mixture was incubated for 24 h at 37 °C. Control proteins were prepared under the same conditions, except that glyoxylic acid was omitted (23). These published methods were modified in order to prepare proteins with a range of CML modifications as follows: protein was incubated in buffer exactly as above, except that varying amounts of glyoxylic acid were added as follows: 10 mg (0.108 M), 7 mg (0.076 M), 4 mg (0.043 M), 2 mg (0.0217 M), and 1 mg (0.018 M); concomitantly varying amounts of sodium cyanoborohydride were added as follows: 19.8 mg (0.315 M), 14.1 mg (0.225 M), 7.1 mg (0.1125 M), 3.5 mg (0.0556 M), and 2 mg (0.0318 M), respectively, as described previously. Preparations of CML-modified proteins were extensively dialyzed against phosphate-buffered saline (PBS) and characterized by percent modification as determined both by employment of 2,4,6-trinitrobenzene sulfonic acid to determine the difference in lysine residues of modified versus unmodified preparations (42) and by gas chromatography–mass spectroscopy (43). To test CML-modified adducts, 0.8 mmol of albumin (BSA), or ovalbumin (OVA) (Sigma) (176 mg in each case), and sodium cyanoborohydride (28.3 mg; 0.49 μM) were dissolved in sodium phosphate buffer (0.2 M, pH 7.8); to this was added glyoxylic acid (14.3 mg; 0.155 M) in a total volume of 1 ml per reaction. The mixture was incubated for 24 h at 37 °C. Control proteins were prepared under the same conditions, except that glyoxylic acid was omitted (23). These published methods were modified in order to prepare proteins with a range of CML modifications as follows: protein was incubated in buffer exactly as above, except that varying amounts of glyoxylic acid were added as follows: 10 mg (0.108 M), 7 mg (0.076 M), 4 mg (0.043 M), 2 mg (0.0217 M), and 1 mg (0.018 M); concomitantly varying amounts of sodium cyanoborohydride were added as follows: 19.8 mg (0.315 M), 14.1 mg (0.225 M), 7.1 mg (0.1125 M), 3.5 mg (0.0556 M), and 2 mg (0.0318 M), respectively, as described previously. Preparations of CML-modified proteins were extensively dialyzed against phosphate-buffered saline (PBS) and characterized by percent modification as determined both by employment of 2,4,6-trinitrobenzene sulfonic acid to determine the difference in lysine residues of modified versus unmodified preparations (42) and by gas chromatography–mass spectroscopy (43). To test CML-modified adducts, 0.8 mmol of CML/mol of Lys, CML-OVA, 33 mmol of CML/mol of Lys, were diluted 1:40 with native OVA in PBS. All CML-modified native proteins, that bodies/F(β1), fragments, and control proteins were devoid of endotoxin prior to experiments by chromatography onto Detox-iGel columns (Pierce). The level of endotoxin in all protein preparations (concentration range, 2–6 mg/ml) was less than 3 pg/ml (Sigma).

Preparation and Characterization of Antibodies—Anti-human RAGE IgG and affinity purified anti-CML IgG were prepared and characterized as described previously (44 and 24, respectively). In the latter case, CML-BSA and CML-KLH prepared as above were employed to immunize New Zealand White rabbits (24). After 6–12 weeks, serum was obtained and IgG prepared (Pierce). Affinity purification of the IgG fractions was performed as follows: IgG fractions were chromatographed onto Affi-Gel 15 resin (Bio-Rad), which had previously adsorbed KLH. Material that did not adhere to the resin was collected and subsequently chromatographed onto Affi-Gel 15 (Bio-Rad) which had previously been adsorbed CML-BSA. After extensive washing in Tris-buffered saline (Tris, 0.02 M, pH 7.4, and NaCl, 0.1 M) containing Tween 20 (0.05%), fractions were eluted in buffer containing glycine, 0.02 M, pH 2.5. A280 nm of each fraction was determined; positive fractions were immediately neutralized and dialyzed versus Tris-buffered saline. Enzyme-linked immunosorbent assays (ELISA) were performed to chara...
CML Addicts Bind RAGE and Modulate Cellular Properties

(Molecular Probe, Nunc) (5 ng/well) in bicarbonate/carbonate buffer, pH 9.6, and incubated for 16 h at 4 °C. Material in the wells was aspirated, and unoccupied sites were blocked by incubation with PBS containing BSA (1%) for 2 h at 37 °C. Wells were washed twice with PBS containing octyl β-glucoside (0.005%) (Roche Molecular Biochemicals). A radioligand binding assay was performed in duplicate to determine the indicated concentration of [125I]human sRAGE alone or in the presence of a 50-fold molar excess of unlabeled human sRAGE or the indicated competitor for 2 h at 37 °C. The suspension was subjected to centrifugation (Amersham Pharmacia Biotech). Equilibrium binding data were analyzed according to the equation of Klotz and Hurston (45): $B = nK_A/1 + K_A$, where $B$ indicates specifically bound ligand (total binding, $n$ indicates sites/cell, $K$ indicates the dissociation constant, and $A$ indicates free ligand concentration) using nonlinear least squares analysis (Pism; San Diego, CA). Specific binding of CML-BSA to radiolabeled RAGE was further determined by subtraction of nonspecific binding (counts obtained upon binding of radiolabeled sRAGE to immobilized BSA) from that obtained upon binding of radiolabeled sRAGE to immobilized CML-BSA. In the case binding to immobilized BSA, counts were normalized to 100% that observed in the absence of non-specific binding, and incubated with tracer alone, minus nonspecific binding, and incubated with tracer in the presence of excess unlabelled material), $n$ indicates sites/cell, $K$ indicates the dissociation constant, and $A$ indicates free ligand concentration.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—HUVECs, murine macrophage BV-2 cells (47), and rat vascular smooth muscle cells (VSMC; generously provided by Dr. Abraham Rothman) (9) were incubated with the indicated CML-modified or native protein for 6 h at 37 °C in the presence or absence of preincubation with the indicated IgG or sRAGE/control protein for 2 and 1 h, respectively, at 37 °C. In certain cases, transient transfection was performed employing mock, or RAGE tail deletion constructs as above, except that DNA, 0.4 μg/ml medium, was employed. Nuclear extracts were prepared (48) and EMSA was performed employing [32P]-labeled probe for NF-κB (49) as described (8–9). Supershift assays were performed by preincubating nuclear extracts with anti-κB or anti-p65 (Santa Cruz Biotechnology) with nuclear extract for 45 min at room temperature prior to addition of radiolabeled oligonucleotide probe. Relative intensity of the bands was determined by densitometric analysis as above.

In Vivo Studies: Infusion of CML-modified and Native Proteins—BALB/c mice (Charles River), approximately 8 weeks of age, were injected intravenously via the tail vein with CML-modified native protein or lipopolysaccharide. Where indicated, animals were pretreated with the indicated IgG 2 h prior to and at the time of infusion of CML-modified proteins. In other cases, CML-modified protein was infused with excess sRAGE for 1 h prior to infusion. Twelve h later, lungs were rapidly harvested. Lung tissue was homogenized in Tris-buffered saline containing protease inhibitor (Roche Molecular Biochemicals) and subjected to centrifugation at 4 °C for 10 min. Supernatant was centrifuged for 1 h at 4 °C at 40,000 rpm and the pellet dissolved in TBS containing protease inhibitors and octyl β-glucoside (2%) for 4 h at 4 °C. The suspension was subjected to centrifugation for 10 min at 14,000 rpm and supernatant assessed for protein concentration (Bio-Rad). Immunoblotting was performed after electrophoresis of 50 μg of protein/well of SDS-PAGE gels and transfer of gel components to nitrocellulose. Anti-VCAM-1 IgG (0.4 μg/ml) was employed for immunoblotting as above. In other cases, total RNA was isolated using TRIZOL (Life Technologies, Inc.) and the concentration of total DNA determined by absorption at A260nm. Total RNA (30 μg/ lane) was subjected to electrophoresis. After electrophoretic separation on a formaldehyde-agarose gel (0.8%), RNA was transferred onto a GeneScreen hybridization filter (NEN Life Science Products) and linked by a UV cross-linker (Stratagene, La Jolla, CA). The filter was then prehybridized in QuikHyb hybridization buffer (Stratagene) for 30 min, followed by hybridization with rat VCAM-1 cDNA probe (generously provided by Dr. Tucker Collins) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe labeled with [α-32P]dCTP using the random primer labeling system (Stratagene) in the above hybridization buffer for 1 h. After washing twice at room temperature (15 min/wash) with SSC (2 times) containing SDS (0.1%) and then with SSC (0.1 time) containing SDS (0.1%) at 60 °C for 30 min, the filter was subjected to autoradiography at ~80 °C. cDNA for glyceraldehyde-3-phosphate dehydrogenase was used in control studies to normalize counts in the VCAM-1 message by densitometry.

Statistical Analysis—Statistical comparisons were determined using one-way analysis of variance; where indicated, individual comparisons were performed using Student's t test. In all cases, statistical significance was ascribed to the data when $p < 0.05$.

RESULTS

CML Modifications Are Present to Enhanced Degrees in Human Serum Albumin in Diabetes and Renal Failure and Bind RAGE

As a first step in elucidating whether CML modifications occurred in typical proteins such as albumin, we isolated human serum albumin from age-matched subjects with diabetes, renal failure, or controls by chromatography of plasma on Affi.

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Gel blue. Albumin preparations, analyzed by SDS-PAGE and immunoblotting with affinity purified anti-CML IgG, displayed ~2.0- and ~3.3-fold increased CML immunoreactivity of samples from diabetic and renal failure patients, respectively, compared with normal controls (Fig. 1A). These findings were consistent with earlier work indicating enhanced CML accumulation in the serum of subjects with diabetes or renal failure and suggested the relevance of albumin for modification by CML in our studies (24–26).

Thus, CML-BSA was prepared and tested for its ability to bind RAGE. When immobilized onto the wells of plastic dishes, CML-BSA bound $^{125}$I-sRAGE in a dose-dependent manner, with $K_r \approx 76.2 \pm 35$ nM (Fig. 1B). The affinity of CML-modified BSA for RAGE was quite similar to that obtained previously employing heterogeneous AGEs, 61 $\pm$ 23 nM (1). That the observed binding was dependent on RAGE was illustrated by inhibition of $^{125}$I-sRAGE interaction with CML-BSA in the presence of excess sRAGE, anti-CML IgG, or anti-RAGE IgG (Fig. 1C). In contrast, preincubation with preimmune rabbit IgG was without effect (Fig. 1C). A polypeptide backbone for the heterogenous AGEs prepared upon incubation of protein with reducing sugar was CML (22). We thus prepared heterogeneous AGEs by incubation of human IgG with ribose, 0.025M (a relevant concentration of reducing sugar in diabetes) for 6 weeks at 37 °C under aerobic conditions. After retrieval of this material and extensive dialysis to remove unreacted ribose, AGE-IgG demonstrated significant immunoreactivity with affinity purified anti-CML IgG (by ELISA) (data not shown). Upon chromatography of AGE-IgG onto Affi-Gel 10 resin to which had previously been adsorbed human RAGE, material immunoreactive with anti-CML IgG (by ELISA) was eluted in the presence of glycine buffer. In contrast, equal volumes of glycine buffer control lacked immunoreactivity with anti-CML-IgG (data not shown). In radioligand binding assays, eluate from this Affi-Gel 10-sRAGE resin substantially suppressed binding of radiolabeled sRAGE to immobilized CML-BSA; in contrast, glycine buffer control was without effect (Fig. 2). These data thus provided further evidence for the specific interaction of CML-modified adducts with RAGE.

Taken together, these studies indicated that chemically synthesized CML-modified protein, or CML adducts that form...
upon incubation of protein with relevant concentrations of reducing sugar under aerobic conditions, bind RAGE in a specific manner. We next sought to extrapolate these findings to those previously observed in \textit{in vitro} cell culture systems to determine if CML-modified adducts ligated RAGE and modulated cellular properties.

\textbf{CML-modified Adducts Mediate Cellular Activation, in Vitro Analyses}

\textbf{Endothelial Cells—}We previously demonstrated that heterogeneous AGEs, either those prepared \textit{in vitro} or those isolated from human diabetic subjects, modulated endothelial function via engagement of RAGE (14). Since \textit{in vitro}, CML-modified adducts bound RAGE, we sought to determine if these modifications were important components of heterogeneous AGEs in effecting cellular activation.

A central means by which endothelial function is modulated is by early enhanced expression of vascular cell adhesion molecule-1 (VCAM-1), a cell adhesion molecule that mediates binding of mononuclear cells bearing VLA-4 to the vessel wall (50). Incubation of HUVEC with CML-modified ovalbumin (CML-OVA), 310 mmol of CML/mol of Lys, resulted in significantly increased cell surface expression of VCAM-1 compared with incubation with native OVA in the presence of anti-RAGE IgG but not by nonimmune IgG (Fig. 4A, lanes 5 and 6, respectively). That this was dependent on ligation of RAGE was demonstrated by suppression of activation of NF-\(\kappa\)B by CML-OVA in the presence of anti-RAGE IgG but not by nonimmune IgG (Fig. 3D, lanes 3 and 4, respectively). Similar inhibitory effects were noted with excess sRAGE (data not shown).

Taken together, these data suggested that CML-modified proteins were specific AGEs that mediate cellular activation in endothelium, at least in part, via RAGE.

\textbf{Vascular Smooth Muscle Cells—}We next sought to test the hypothesis that CML-modified proteins interacted with RAGE on vascular smooth muscle cells (VSMC), as such cells have been implicated in vascular perturbation in diabetes and renal failure. In VSMC, activation of NF-\(\kappa\)B is a potent means by which regulation of cytokines and vasoregulatory mediators is affected (52–53).

We first tested varied extents of CML modification in order to determine if their interaction with VSMC RAGE resulted in activation of NF-\(\kappa\)B. Incubation of VSMC with CML-BSA (90, 160, or 310 mmol CML/mol Lys) resulted in \(\sim 3.2-\), \(\sim 2.6-\), and \(\sim 3.2\)-fold induction of nuclear translocation of NF-\(\kappa\)B (Fig. 4A, lanes 3–5, respectively) compared with incubation in the presence of native BSA (Fig. 4A, lane 2). In VSMC treated with CML-BSA, 90 mmol of CML/mol of Lys, activation of NF-\(\kappa\)B was dependent on interaction with RAGE, as demonstrated by suppression in the presence of either anti-RAGE IgG or excess sRAGE (Fig. 4B, lanes 5 and 6, respectively). In contrast, nonimmune IgG was without effect (Fig. 4B, lane 4). Furthermore, incubation of VSMC with CML-OVA, 33 mmol of CML/mol of Lys, resulted in \(\sim 3.1\)-fold increase in activation of NF-\(\kappa\)B compared with cells incubated with native OVA (Fig. 4C, lanes 3 and 2, respectively), that these effects were mediated by RAGE was indicated by marked suppression of CML-OVA-mediated activation of NF-\(\kappa\)B in the presence of anti-RAGE IgG (Fig. 4C, lane 5). Similar suppressive effects were noted in the presence of excess sRAGE (data not shown). In contrast, incubation with nonimmune IgG was without effect (Fig. 4C, lane 4).
These data indicated that at low levels of CML modification, interaction of these modified adducts with RAGE on VSMC resulted in activation of NF-κB, thereby providing a potent mechanism by which VSMC function may be altered in the presence of CML adducts of proteins.

Mononuclear Phagocytes—Previous studies from our laboratory indicated that MP properties are significantly modulated upon ligation of RAGE by heterogeneous AGEs, either those prepared in vitro or those derived from in vivo sources, such as AGEs isolated from diabetic plasma, or AGE-β2-microglobulin isolated and purified from the urine/plasma of subjects with DRA (16). It was thus important to determine if CML adducts, present in these preparations, could activate RAGE on MPs causing cellular migration and activation in this setting (54). Furthermore, since recent studies have indicated that CML modifications may result not only from glycation/oxidation of proteins, but also secondary to modification of lipids, a possible role CML-RAGE interaction in lipid-rich foam cells and atherosclerotic plaques, even in euglycemia, might occur (5).

We thus tested the ability of CML-modified adducts to activate monocytes. In modified chemotaxis chambers, compared with native OVA, addition of CML-OVA, 310 mmol of CML/mol of Lys, resulted in increased migration of Molt-4 cells, mononuclear type cells that bear cell surface RAGE (Fig. 5 A, lines 1 and 2, respectively). The effects of CML-OVA were mediated by cellular RAGE as preincubation of Molt-4 cells with anti-RAGE F(ab’2) resulted in suppression of migration in a dose-dependent manner (Fig. 5 A, lines 4 and 5); preincubation with nonimmune F(ab’2) was without effect (Fig. 5 A, line 3). Similarly, preincubation of CML-OVA with excess sRAGE resulted in dose-dependent suppression of Molt-4 migration (Fig. 5 A, lines 6 and 7). Intact RAGE signaling was required for these CML-mediated effects on migration as transient transfection of Molt-4 cells with a construct expressing tail deletion RAGE resulted in significant suppression of Molt-4 migration in response to CML-OVA; mock transfection was without effect (Fig. 5 A, lines 9 and 8, respectively).

It was important to test lower extents of modification of CML-OVA in order to determine their ability to modulate Molt-4 migration. These studies revealed that addition of CML-OVA, 33 mmol/mol Lys, to chemotaxis chambers resulted in increased migration of Molt-4 cells compared with native OVA.
FIG. 4. Ligation of RAGE by CML adducts activates NF-κB in vascular smooth muscle cells. A—C, EMSA. Rat VSMC were treated with the indicated mediators for 6 h. A, VSMC were treated with CML-BSA, 90, 160, or 310 mmol of CML/mol of Lys (lanes 3–5). B, VSMC were treated with CML-BSA, 90 mmol of CML/mol of Lys in the presence of anti-RAGE IgG, nonimmune IgG, or excess sRAGE as above. C, VSMC were treated with CML-OVA, 33 mmol of CML/mol of Lys, in the presence of anti-RAGE IgG or nonimmune IgG. In all cases, after incubation as indicated, nuclear extracts were prepared and EMSA performed employing radiolabeled probes for NF-κB. Lanes designated excess unlabeled NF-κB indicate that a 100-fold molar excess of unlabeled NF-κB probe was added to incubation mixtures of nuclear extracts from CML-adduct-treated cells. Results of densitometric analysis after normalization to native OVA or native BSA, arbitrarily defined as “1” in these experiments, are shown. EMSA were performed three times with analogous results.

(FIG. 5B, lines 2 and 1, respectively). These effects were mediated by ligation of RAGE as demonstrated by suppression of migration in the presence of either anti-RAGE F(ab)_2 or excess sRAGE (Fig. 5B, lines 4 and 5, respectively). In contrast, incubation with nonimmune F(ab)_2 was without effect (Fig. 5B, line 3).

Recent reports have indicated that in lens protein and skin collagen retrieved from elderly human subjects, extent of modification by CML adducts is in the approximate range of 4.95 and 1.70 mmol of CML/mol of Lys, respectively (51). The extent of CML modification is likely to be higher in atherosclerotic disease, as shown in older age groups (53, 54). The increased extent of CML modification is likely to be higher in atherosclerotic plaque tissue (51). Consistent with these findings, exposure of MP-like BV2 macrophages to CML-OVA resulted in ~3.5-fold increase in nuclear translocation of NF-κB by EMSA, compared with incubation in the presence of native OVA (Fig. 5D, lanes 1 and 2). Activation of NF-κB by CML adducts was due to ligation of RAGE as exemplified by significant suppression of NF-κB in the presence of anti-RAGE IgG, 70 µg/ml (Fig. 5D, lane 6), but not in the presence of either lower concentrations of anti-RAGE IgG, 0.7 µg/ml, or nonimmune IgG (Fig. 5D, lanes 5 and 4, respectively). Supershift assays with anti-p50 and anti-p65 IgG demonstrated that the NF-κB complex activated upon ligation of BV-2 RAGE by CML-OVA was composed of both p50 and p65 (Fig. 5D, lanes 8–10). Similar inhibitory effects were observed in the presence of transient transfection of construct encoding DN-RAGE (Fig. 5E, lane 2) or excess sRAGE (data not shown) but not by nonimmune IgG (Fig. 5E, line 3).

CML-modified Adducts Mediate Cellular Activation, in Vivo Analyses

The results of in vitro analyses suggested that CML adducts, at physiologically relevant levels of modification, were capable of mediating cellular activation in a range of cell types. A central test of this hypothesis was the ability of CML-modified adducts to modulate cellular properties in vivo. As a first test of these concepts, we infused CML-BSA, 310 mmol of CML/mol of Lys, into immunocompetent, non-diabetic mice. Initial studies indicated that in comparison to infusion of native BSA, CML-BSA induced a ~46-fold increase in mRNA for VCAM-1 in lung tissue (Fig. 6A, lanes 2 and 1, respectively). To determine if this was associated with modulation of VCAM-1 protein levels in the lung, immunoblotting was performed. Indeed, infusion of CML-BSA into mice resulted in a ~3.6-fold increase in expression of VCAM-1 protein compared with infusion of native BSA (Fig. 6B, lanes 2 and 3, respectively). That this was dependent on ligation of vascular RAGE in the lung by CML-BSA was demonstrated by experiments in which mice were pre-infused with either anti-RAGE IgG or excess sRAGE; in both cases, significant suppression of CML-mediated expression of VCAM-1 in the lung was noted (Fig. 6B, lanes 4 and 6, respectively). In contrast, infusion of nonimmune IgG had no effect on CML-mediated modulation of VCAM-1 expression (Fig. 6B,
These findings illustrate a mechanism by which engagement of vascular RAGE by CML-modified adducts enhances expression of VCAM-1 and, likely, increases adherence of proinflammatory mononuclear cells to the vessel wall.

**DISCUSSION**

Increased recognition of specific AGEs and the multiple means by which they might form in diverse settings such as hyperglycemia, renal failure, and inflammation has highlighted the importance of determining mechanisms by which each might modulate cellular properties, either by receptor-dependent or -independent pathways. In this context, previous studies indicated that heterogenous AGEs, especially those derived from *in vivo* sources, were signal-transducing ligands of RAGE. Specifically, interaction of AGE-β2-microglobulin, isolated from the urine of subjects with renal failure and DRA, with cellular RAGE resulted in enhanced MP migration and generation of proinflammatory cytokines such as tumor necrosis factor-α (16). Since recent studies have shown AGE-β2M itself to be quite heterogeneous, composed at least in part of AGEs such as pentosidine, CML, and imidizalone (55–57), it was important to begin to delineate those specific AGEs that interacted with RAGE.

In the present work, we have identified that CML adducts, dominant AGEs within the tissues, are signal-transducing ligands for RAGE in vitro, in endothelial cells, mononuclear phagocytes, and vascular smooth muscle, and *in vivo*, upon infusion into mice. Importantly, we tested varied extents of CML modification to demonstrate that the CML adducts thus formed exerted specific and receptor-dependent effects. In this context, physiologically relevant levels of CML modification of ovalbumin were capable of modulating MP function. Such levels of modification parallel those reported in relatively inert tissue derived from elderly human subjects, such as skin collagen and lens protein (51). We speculate that in a highly biologically active milieu, such as lipid-laden atherosclerotic fatty streaks and plaques and chronically inflamed joints, levels of CML modification will likely exceed those tested here. In proper context, however, levels of CML-modified adducts are low, even in tissue from subjects with diabetes or renal failure (51). We hypothesize that the inexorable accumulation of AGEs, via their interaction with RAGE, causes a change in cellular homeostasis priming mechanisms capable of triggering full-blown cellular activation. Such chronic AGE engagement of RAGE causes a shift in the basal state, favoring cellular activation with subsequent environmental challenge. Thus, AGE-RAGE interaction may be considered the first hit in a “two-hit”

**FIG. 5.** Ligation of RAGE by CML adducts activates mononuclear phagocytes. A—C, modified chemotaxis assays. The indicated CML-OVA modifications were placed in the lower chamber of modified chemotaxis chambers, and human Molt-4 cells, which bear cell surface RAGE, were placed in the upper chamber for 4 h in the presence or absence of the indicated treatment as described above. Cells that had migrated through the membranes were stained and counted. Extents of modification by CML in OVA were tested as follows: 310 mmol of CML/mol of Lys (A); 33 mmol of CML/mol of Lys (B); and 0.8 mmol of CML/mol of Lys (C). The mean ± S.E. of triplicate determinations is reported. These experiments were performed at least four times with analogous results. D and E, EMSA. Murine BV-2 macrophages were treated with CML-OVA, 310 mmol of CML/mol of Lys or native OVA for 6 h. D, cells were pretreated with anti-RAGE IgG or nonimmune IgG as indicated prior to incubation with CML-OVA. Supershift assays were performed on nuclear extract of CML-treated BV2 cells with the indicated antibody(ies). E, certain BV-2 cells were transiently transfected with a construct encoding DN RAGE (RAGE tail deletion) or mock (vector alone). In all cases, after incubation as indicated, nuclear extracts were prepared and EMSA performed employing radiolabeled probes for NF-κB. Lanes designated *excess unlabeled NF-κB* indicate that a 100-fold molar excess of unlabeled NF-κB probe was added to incubation mixtures of nuclear extracts from CML-adduct-treated cells. Results of densitometric analysis are reported after normalization to native OVA (D) or CML-OVA (E)-treated BV-2 cells, arbitrarily defined as “1” in these experiments. EMSA were performed three times with analogous results.
model of diabetic complications. In wound repair, the second hit comprises the presence of foreign bodies and bacterial pathogens. In atherosclerotic vasculature, the second hit is an environment enriched in modified lipoproteins and macrophages. Juxtaposition of these two mechanisms results in an exaggerated cellular response, ultimately compromising reparative processes in diabetic tissues. Thus, as opposed to septic shock, in which highly potent lipopolysaccharide released by Gram-negative bacteria engages cellular sites, resulting in striking rapid activation and destabilization of homeostasis, in settings such as diabetes, renal failure, and chronic arthritis, long term, yet unrelenting, chronic cellular activation ensues, eventuating in irreversible tissue injury.

An important means by which AGEs modulate cellular properties is via generation of enhanced oxidant stress, manifested by increased transcripts for heme oxygenase and nuclear translocation of NF-κB (8, 9), the latter a central cell signaling molecule whose activation is linked to transcriptional regulation of a range of proinflammatory genes, including RAGE itself (58).

We speculate that these events, significantly suppressed in the presence of antioxidants (8, 9) and mediated at least in part via RAGE, are early and highly influential steps in processes which, long term, contribute to cellular dysfunction. Consistent with these concepts, in experimental diabetes, AGEs begin to form within weeks of hyperglycemia (Refs. 19, 20, and 59). Thus, although evidence does not exist for globally enhanced formation within weeks of hyperglycemia (Refs. 19, 20, and 59). Thus, although evidence does not exist for globally enhanced oxidant stress in diabetes, numerous factors likely converge in the development of cellular abnormalities. In diabetes, hyperglycemia directly results in activation of protein kinase C, especially the ε isoform, leading to cellular dysfunction (73–74). In addition, accelerated generation and accumulation of products of the sorbitol/polyol pathway are implicated in the pathogenesis of diabetic complications, especially those of the retina and peripheral nerve (75). Recent observations suggesting that polyol metabolites may lead to generation of AGEs (76) suggest that, not unexpectedly, seemingly diverse pathways may converge and thus collaboratively contribute to cellular perturbation. These findings indeed parallel the diverse means by which...
CML adducts may form from glycation/oxidation of proteins to lipid oxidation and myeloperoxidase-driven modification of amino acids and related structures.

Taken together, our observation that physiologically relevant extents of CML modification in proteins are signal-transducing ligands for RAGE transforms these adducts from inert biomarkers of aging and chronic disease to bioactive species capable of altering properties of cells critically perturbed in inflammatory milieu. Our findings provide a contributory mechanism for the chronic, yet unrelenting progressive vascular and inflammatory cell dysfunction that typifies the complications of diverse disorders, such as diabetes, renal failure, chronic inflammation and Alzheimer's disease, in which CML adducts accumulate and the expression of RAGE is enhanced. Thus, identification of specific molecular structures to inhibit interaction of CML-modified adducts with RAGE may provide a potent means to suppress cellular activation and, thereby, limit long term tissue injury in chronic diseases.

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$N^\epsilon$-(Carboxymethyl)Lysine Adducts of Proteins Are Ligands for Receptor for Advanced Glycation End Products That Activate Cell Signaling Pathways and Modulate Gene Expression

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