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N^{ε}-(Carboxymethyl)lysine induces γ-glutamylcysteine synthetase in RAW264.7 cells

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Abstract

Advanced glycation end products (AGEs) play an important role in the development of angiopathy in diabetes mellitus and atherosclerosis. Here, we show that adducts of N^{ε}-(carboxymethyl)lysine (CML), a major AGE, and bovine serum albumin (CML-BSA) stimulated gamma-glutamylcysteine synthetase (γ-GCS), which is a key enzyme of glutathione (GSH) synthesis, in RAW264.7 mouse macrophage-like cells. CML-BSA stimulated the expression of γ-GCS heavy subunit (h) time- and dose-dependently and concomitantly increased GSH levels. CML-BSA also stimulated DNA-binding activity of activator protein-1 (AP-1) within 3 h, but the stimulatory effect decreased in 5 h, and nuclear factor-κB (NF-κB) with a peak activity at 1 h and the stimulatory effect diminished in 3 h. Studies of luciferase activity of the γ-GCSh promoter showed that deletion and mutagenesis of the AP-1-site abolished CML-BSA-induced up-regulation, while that of NF-κB-site did not affect CML-BSA-induced activity. CML-BSA also stimulated the activity of protein kinase C, Ras/Raf-1, and MEK/ERK1/2. Inhibition of ERK1/2 abolished CML-BSA-stimulated AP-1 DNA-binding activity and γ-GCSh mRNA expression. Our results suggest that induction of γ-GCS by CML adducts seems to increase the defense potential of cells against oxidative stress produced during glycation processes.

Keywords: AGE; CML; Glutathione; γ-glutamylcysteine synthetase; RAW264.7 cells

Diabetes mellitus (DM) is a common condition with associated with various complications, such as retinopathy, neuropathy, nephropathy, and vasculopathy. Advanced glycation end products (AGEs), which are derivatives of nonenzymatic reactions between sugars and proteins or lipids, are closely related to hyperglycemia and are observed in various DM-related complications [1]. In vitro studies have shown that AGEs are part of complex interactions within oxidative stress and vascular damage in atherosclerosis [2,3]. AGEs are a heterogeneous class of compounds and taken up through receptors, such as AGE-specific cell surface receptors (RAGEs) [4], macrophage scavenger receptor-A [5], and galectin-3 [6]. These receptor-mediated cell signals induce gene expressions [4,5].

N^{ε}-(carboxymethyl)lysine (CML)-modified proteins are important AGEs in vivo. They accumulate especially in vascular tissue, atherosclerotic lesions, and glomerular tissue retrieved from diabetic rodents [4,7–11]. Recently, Kislinger et al. [4] reported that CML adducts are ligands for RAGE and activate nuclear factor-κB (NF-κB) to modulate gene expression. This is the first report on the receptor-mediated signal pathways by CML adducts.

Glutathione (γ-glutamylcysteinyl glycine, GSH), participates in many biological processes especially cellular defense against oxidative stress induced by reactive oxygen species (ROS) [12]. GSH is synthesized via two ATP-requiring steps catalyzed by γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme for the

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synthesis of GSH and GSH synthetase. The activity of γ-GCS is important for maintaining adequate levels of GSH. γ-GCS consists of a heavy subunit (γ-GCSh), which is catalytic, and a light subunit, which is regulatory [13–15]. Recent studies suggested that one of the most important roles of intracellular GSH is redox regulation. Redox state controls intracellular signal pathways, DNA transactivation, protein folding, chaperone activity, and enzyme function [16].

We have previously reported that the presence of high glucose concentrations is associated with low levels of GSH in vascular endothelial cells and embryos [17,18], and impairment of redox-regulated intracellular signal pathways [17]. In this regard, the importance of GSH in regulating cellular function in DM is not clear at present.

In the present study, we investigated the transcriptional regulation of γ-GCS expression by CML adducts in macrophage cells, which play a role in the progression of vascular injury. Our results showed that CML adducts induce γ-GCS mainly by activator protein (AP)-1 through protein kinase C (PKC)/Ras/Raf-1/extracellular signal-regulated kinases (ERK)1/2 pathway.

Research design and methods

Materials. RAW264.7 mouse macrophage cells were purchased from the Health Sciences Research Resources Bank (Tokyo, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2 and 100% humidity. CML-BSA was prepared as described previously [19]. Briefly, 2 mg/ml of BSA was incubated at 37 °C for 24 h with 0.75 mol/l glyoxylic acid and 0.3 mol/l $NaCNBH_3$ in 0.5 mol/l sodium phosphate buffer, pH 7.4. This was followed by dialysis against 1000 volume of phosphate buffered saline for two times. In this CML-BSA preparation, the extent of modification was 34.5 mol of CML/mol of BSA. As a control, BSA was incubated at 37 °C for 24 h without 0.75 mol/l glyoxylic acid and 0.3 mol/l $NaCNBH_3$ in 0.5 mol/l sodium phosphate buffer, pH 7.4.

Estimation of GSH. The level of GSH was estimated enzymatically as described by Beutler [20].

Western blots. Lysate from the extract of 1×10^5 cells was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in a 7.5% gel, transferred to a nitrocellulose membrane, and immunologically stained using rabbit anti-human γ-GCSh IgG as the first antibody, and then, with horseradish peroxidase-labeled anti-rabbit IgG as the second antibody. Blots were developed by enhanced chemiluminescence using the ECL kit and the relative immunological activity was analyzed by NIH image. The protein concentration was determined according to [21], with bovine serum albumin (BSA) as the standard.

Northern blots. γ-GCSh cDNA was prepared as described previously [22]. The cloned cDNA was isolated as described by Godwin et al. [23]. Isolation of cytoplasmic RNA and Northern blotting were essentially performed as described by Sambrook et al. [24]. Cytoplasmic RNAs isolated from RAW264.7 cells were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde, subsequently transferred to nylon membranes, and then hybridized with ^{32}P-labeled γ-GCSh probe. Autoradiographed membranes were analyzed using a Fujix Bio-Analyzer BAS-5000 (Fuji Photo Film, Tokyo). After being stripped, the membranes were rehybridized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The relative radioactivity was expressed as a ratio of photostimulated luminescence (PSL) corrected by the intensity of GAPDH.

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Abmays and Warkman [25]. Briefly, the cells were suspended in hypotonic buffer, 10 mM HEPES, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM dithiothreitol (DTT). The swollen cells were homogenized and the nuclei pelleted. Soluble nuclear proteins were prepared by adding a high-salt buffer, 20 mM HEPES, pH 7.9, containing 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT followed by centrifugation.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay for AP-1 and NF-κB was performed as described by Sen and Baltimore [26] with a slight modification. Briefly, nuclear extracts were incubated with ^{32}P-oligonucleotides specific for AP-1or NF-κB. The binding reaction proceeded in a 20-μl reaction mixture containing 10 μg of extract, 4 μl of a binding buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 4% glycerol), 2 μg of poly(dI-dC) as a non-specific competitor DNA, 2 μg of bovine serum BSA and labeled oligonucleotide (3000–6000 cpm). After a 30-min binding reaction at room temperature, samples were loaded on a 6% nondenaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris, 45 mM borate, and 0.5 mM EDTA, pH 8.0. As a specificity control, a 100-fold excess of unlabeled probe was applied. The sequence of the binding was prepared according to the nucleotide sequence of the human γ-GCSh promoter region containing 5′-TGATTCA-3′ for the AP-1 probe, 5′-GGGATTCCC-3′ for the NF-κB probe, respectively. The DNA-binding activity of the extracts was quantified by estimating the amount of the DNA complexes with ^{32}P-labeled AP-1and NF-κB excised from the dried gels and was expressed as PSL.

Luciferase assay of γ-GCSh promoter. The human γ-GCSh promoter and 5′-deletion constructs were used for the transfection of RAW264.7 cells. Synthetic oligonucleotides (20-mer) were prepared based on the published DNA sequence of the γ-GCSh promoter region as described previously [27]. The reporter plasmids for expression in RAW264.7 cells were constructed by sub-cloning a *Sac*l fragment of approximately 2.2 kbp of the human γ-GCSh promoter gene, into a *Sma*l site of pGL3 luciferase vector (PGL1 vector) (Promega, Madison, WI). To generate the deletion constructs for NF-κB (−1099:−1091), the γ-GCSh promoter template was deleted using *Kpn*l (PGL2 vector). To mutate the AP-1-like element (−280:−253), site-directed mutagenesis of AP-1 was constructed using a LAPCR in vitro Mutagenesis Kit (Takara, Tokyo). 5′-TGATTCA-3′ was modified to 5′-TGATTTG-3′ (PGL AP-1 mut. vector). For the NF-κB site, 5′-GG AAATCCC-3′ was modified to 5′-CTCAATCCC-3′ (PGL NF-κB mut. vector). Sub-confluent cultures of RAW264.7 cells were transfected using 5 μg/ml of Lipofectin reagent (Life Technologies, Gaithersburg, MD), 2 μg/ml of pGL3 luciferase reporter vector to construct various γ-GCSh promoters and 0.5 μg/ml PRL-TK control vector (Promega) as an internal control to normalize transfection efficiency. Before transfection, the growth medium was replaced with Opti-MEM (Life Technologies). After transfection for 16 h at 37 °C in 5% CO_2 under 100% humidity, Opti-MEM was replaced with normal growth medium. Forty-eight hours after the beginning of transfection, cells were treated with 100 μg/ml of CML-BSA for 12 h, then harvested using passive lysis buffer (Promega). Crude cell lysates were centrifuged to remove debris and kept frozen at −80 °C until use. Luciferase activity was measured using the Dual-Luciferase Assay System (Promega) and a luminometer. Data of firefly luciferase chemiluminescence were standardized by renilla luciferase chemiluminescence values.

Immune complex kinase assay. The phosphorylation of a mitogen-activated protein kinase (MAPK) such as the extracellular signal-regulated kinases 1 and 2 (ERK1/2, p44/p42), c-Jun N-terminal kinase (JNK, p54/p46), and $p38^{MAPK}$, was measured as described in the protocol supplied by New England BioLabs (Beverly, MA), using phospho-specific antibodies against phosphorylated sites of ERK1/2, JNK, and $p38^{MAPK}$. The phosphorylated forms of proteins were detected by ECL chemiluminescence. Raf-1 kinase activity was measured using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Bucking-

hamshire, UK) and Purified MEK-1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) according to the method described by Egea et al. [28]. Briefly, cells were washed twice with ice-cold PBS and lysed in Triton lysis buffer (25 mM Tris, pH 8.0, 137 mM NaCl, 1% Triton X-100, and 10% glycerol) containing protease and phosphatase inhibitors (PMSF/aprotinin/pepstatin and sodium orthovanadate/sodium fluoride). For kinase assay, cell extracts (50 μg) were incubated with 0.5 μg of antibody against Raf-1 (Santa Cruz Biotechnology). After incubation for 2 h at 4 °C, 30 μl of protein A–agarose was added and incubated for an additional 30 min. Raf immunocomplexes were washed three times with Triton lysis buffer and twice with MEK buffer (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 5 μM ATP). Immune complexes were then incubated in 40 μl of kinase buffer containing 20 μCi [γ-^{32}P]ATP and 0.5 μg of MEK-1 for 30 min at room temperature. Reactions were terminated by the addition of 40 μl of 2× Laemmli sample buffer and boiling for 5 min. Reaction products were separated by SDS–PAGE (10% gel). After drying the gel, the phosphorylation signal was analyzed using BAS5000 (Fuji Photo Film).

Assay for translocation of PKC. Cells were left untreated or were stimulated with 100 μg/ml of CML-BSA. The cells were washed and sonicated in ice-cold homogenization buffer [20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 50 mM 2-ME, 0.5 mM PMSF, 100 μg/ml leupeptin, and 10 μg/ml aprotinin] and then separated into cytosolic and membrane fractions by centrifugation (45,000*g*, 30 min). Membrane extracts were obtained by shaking the membrane fraction in ice-cold washing buffer [20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 50 mM 2-ME, 0.5 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1% Triton X-100] followed by centrifugation. Measurement of PKC activity in each fraction was performed using a PKC enzyme assay system (Amersham, Arlington Heights, IL). Protein concentrations were determined using BCA Kit (Pierce).

Statistical analysis. Data are expressed as means ± SD. Differences between groups were examined for statistical significance by Student's two-tailed *t* test, or by one-way Fractional ANOVA test. Significance was taken as $p < 0.05$ for Student's *t* test and $p < 0.0001$ for Fractional ANOVA test, respectively.

Results

CML-BSA increases GSH levels and induces γ-GCSh mRNA expression

In the first step, we determined the levels of GSH and expression of γ-GCSh mRNA and studied the effects of CML-BSA using RAW264.7 mouse macrophage-like cells. The addition of CML-BSA at 100 μg/ml increased GSH levels by 2.1-fold in 12 h (control: 11.7 ± 1.3 nmol/10^6 cells, CML-BSA: 24.6 ± 1.5 nmol/10^6 cells) (Fig. 1A), while no increase in GSH level was noted by the addition of non-glycated BSA. CML-BSA also induced the expression of γ-GCSh mRNA, as demonstrated on Northern blot analysis (Fig. 1B). Considering the relative intensity of γ-GCSh mRNA in the cells before treatment as 100%, that in cells treated with 100 μg/ml of CML-BSA for 6 h was 205 ± 2.5% (mean of three independent analyses). In contrast, non-modified BSA did not induce γ-GCSh mRNA. Further studies of immunoblot analysis showed that CML-BSA also induced γ-GCSh protein (Fig. 1C). In the next step, we examined the effect of incubation time and dose of CML-BSA on γ-GCSh mRNA levels. The time course study showed that the expression of γ-GCSh mRNA increased within 1 h of treatment with 100 μg/ml of CML-BSA, and the peak level was noted at 6 h but the level diminished subsequently at 12 h (Fig. 1D). Furthermore, the effect of CML-BSA on γ-GCSh mRNA expression was dose-dependent (Fig. 1E).

CML-BSA stimulates AP-1 and NF-κB activities

AP-1 and NF-κB-binding sites are present in the 5′-flanking region of γ-GCSh mRNA. Fig. 2A shows the results of electrophoretic mobility shift assay for AP-1 of the γ-GCSh promoter. Treatment of cells with 100 μg/ml of CML-BSA stimulated AP-1-DNA binding activity with a peak at 3 h but the stimulatory effect decreased in 5 h (Fig. 2A, lanes 4–6). Electrophoretic mobility shift assay for NF-κB of the γ-GCSh promoter showed that CML-BSA also stimulated NF-κB activity with a peak at 1 h and the stimulatory effect diminished in 3 h (Fig. 2B), findings that are in good agreement with the previous data [4].

CML-BSA stimulates transcription of γ-GCSh gene

Next, we constructed chimeric genes containing various regions of the γ-GCSh gene promoter and RAW264.7 cells were transiently transfected with pGL3 basic vector containing the γ-GCSh promoter construct. Luciferase activity stimulated by 100 μg/ml of CML-BSA was found in the γ-GCSh promoter containing the NF-κB and AP-1 binding sites (Fig. 3). Deletion and mutagenesis of the NF-κB site did not reduce the basal luciferase activity and slightly decreased CML-BSA-dependent luciferase activity. Depletion and mutagenesis of AP-1 sites largely decreased the CML-BSA-dependent activity. These results strongly suggest that CML-BSA stimulation of the transcription of this gene is mediated mainly by AP-1 under the conditions employed.

CML-BSA stimulates PKC, Raf-1, and ERK1/2 activities

To determine the signal pathways of CML-BSA-induced γ-GCSh, we measured the activity of PKC, Raf-1, and ERK1/2. CML-BSA stimulated the activity of PKC (Fig. 4A, left), Raf-1 (Fig. 4B), and ERK1/2 (Fig. 4C). In contrast, CML-BSA did not stimulate JNK or $p38^{MAPK}$. H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride], a specific inhibitor of PKC, inhibited the up-regulation of γ-GCSh protein stimulated by CML-BSA (Fig. 4A, right). PD98059 [2-(2′-amino-3′-methylphenyl)oxanaphthalene-4-one], a specific inhibitor of ERK1/2, inhibited the phosphorylation of ERK1/2 (Fig. 5A). The MEK 1 inhibitor also abrogated CML-BSA-stimulated AP-1-DNA binding activity (Fig. 5B) and γ-GCSh mRNA (Fig. 5C).

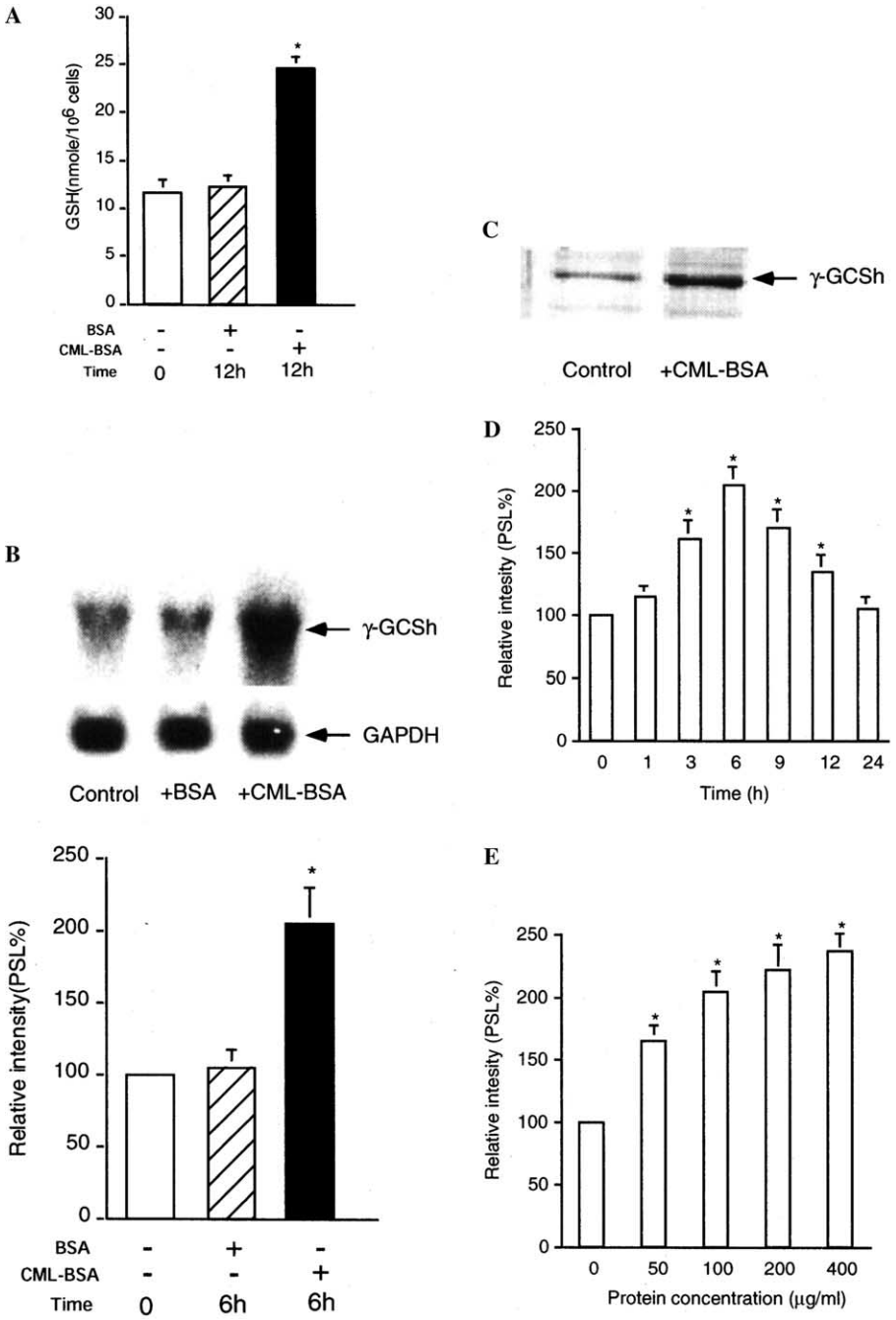

Fig. 1. CML-BSA induces GSH and γ-GCSh. RAW264.7 cells were treated with 100 μg/ml of non-treated BSA (lane 2), 100 μg/ml of CML-BSA (lane 3), and RNAs were prepared after 6 h. The level of GSH was estimated enzymatically (A), the expression of γ-GCSh mRNA from Northern blots (B) and γ-GCSh protein from Western blots (C). The effect of incubation time (D), and dose-dependent effect of CML-BSA (E) on the expression of γ-GCSh mRNA were estimated in RAW264.7 cells after 6 h. Values were normalized to the GAPDH mRNA level and are expressed as relative intensity (PSL%) with the steady state levels of γ-GCSh in RAW264.7 cells as 100%. Data are means ± SD of three independent studies. $^{*}p < 0.01$ vs each control.

Discussion

The removal of AGE-modified proteins is facilitated through cell surface receptors in murine RAW264.7 macrophage-like cells [29,30]. In addition to the uptake and degradation of AGEs by macrophages, interactions between AGE-ligands and receptors induce a range of biologically important responses, including chemotaxis [31], cell activation, and cytokine and growth factor secretion [32–34]. In macrophages, AGEs are known to induce tumor necrosis factor (TNF)-α, interleukin (IL)-1, platelet derived growth factor, and insulin-like growth factor-1 [34]. However, the signal cascade(s) for these gene expressions by AGEs is not clear at present.

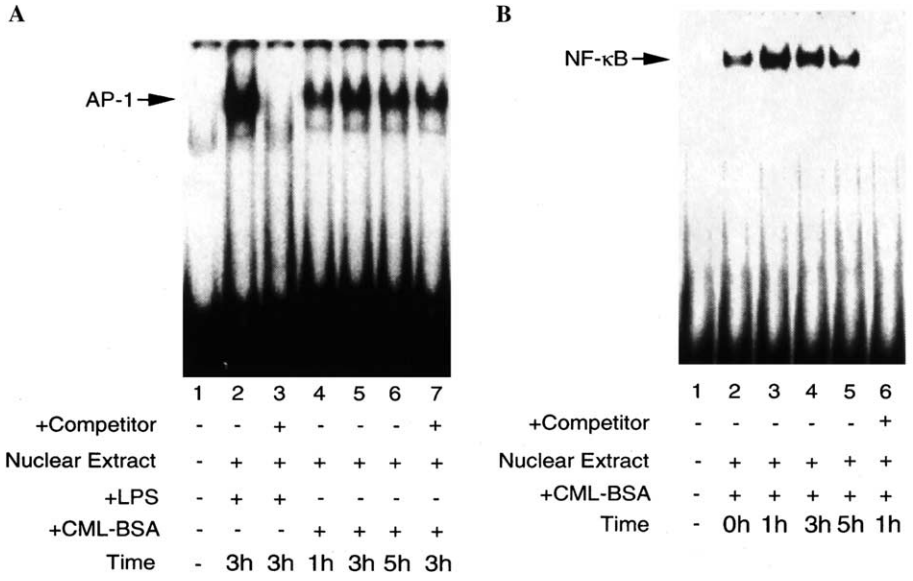

Fig. 2. Electrophoretic mobility shift assay of AP-1 and NF-κB. RAW264.7 cells were treated with CML-BSA and nuclear extracts drawn at the indicated times were incubated with an AP-1 or NF-κB-specific ^{32}P-oligonucleotide for 30 min and then loaded on a 6% nondenaturing polyacrylamide gel. The DNA binding activities of the extracts were estimated by electrophoretic mobility shift assay. (A) AP-1 activity; lane 1, free probe; lanes 2–7, cell-nuclear extracts from RAW264.7 cells; lanes 2 and 3, +100 ng/ml of lipopolysaccharide (LPS) as a positive control; lanes 3 and 7, +competitor for AP-1; lanes 4–7, +100 μg/ml of CML-BSA for 1 h (lane 4), 3 h (lane 5), 5 h (lane 6), and 3 h (lane 7). (B), NF-κB activity; lanes 1, free probe; lanes 2–6, + CML-BSA for 0 h (lane 2), 1 h (lanes 3 and 6), 3 h (lane 4), and 5 h (lane 5); lane 6, lane 3 + competitor.

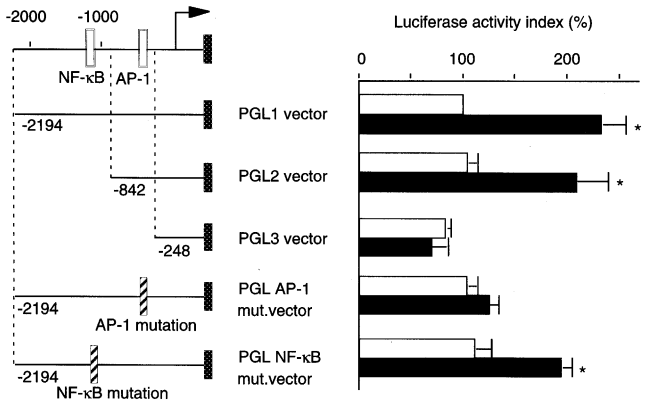

Fig. 3. Luciferase activity of γ-GCSh promoter. RAW264.7 cells were treated with 100 μg/ml of CML-BSA and incubated for 12 h. Putative sequences in the 5′-upstream region of the human γ-GCSh gene and restricting enzymes used are shown (upper left). Numbers indicate the distance in base pairs from the start of transcription. PGL1 vector, a *Sac*I fragment of γ-GCSh promoter; PGL2 vector, PGL1 vector lacking NF-κB; PGL3 vector, PGL1 vector lacking NF-κB and AP-1; PGL AP-1 mut vector, pGL1 vector with a mutation at the site AP-1; PGL NF-κB mut vector, PGL1 vector with a mutation at the NF-κB site. Data of firefly luciferase chemiluminescence were standardized by renilla luciferase chemiluminescence values. Luciferase activity index was expressed as standardized data of PGL1 vector in the absence of CML-BSA as 100%. Data are means ± SD (%) of three independent studies. $^{*}p < 0.01$.

Kislinger et al. [4] has reported the role of CML adducts in intracellular signaling pathways and the modulation of gene expression. One pathway of RAGE-dependent cellular perturbation involves activation of p21^{ras}, followed by activation of MAPKs and NF-κB, resulting in transcription of target genes [35–37]. On the other hand, Huttunen et al. [38] has reported that RAGEs mediate distinct signals to stimulate NF-κB activity and induce neurite outgrowth. Still, the pathological significance of the activation of intracellular signal cascades by AGEs remains to be clarified.

In the present study, we demonstrated that CML-BSA stimulated the expression of γ-GCSh mRNA (Fig. 1). It has been reported that the levels of stress-responsive transcription factors such as AP-1, AP-2, and NF-κB affect the expression of γ-GCSh [39]. NF-κB is also considered to induce the expression of γ-GCSh in hypoxia [40]. In the present study, AP-1-DNA binding activity as well as NF-κB activity was stimulated by CML-BSA as demonstrated in electrophoretic mobility shift assays (Fig. 2). Interestingly, studies of luciferase activity for the γ-GCSh promoter (Fig. 3) showed that deletion and mutagenesis of the AP-1-site abolished CML-BSA-induced up-regulation, while that of NF-κB-site did not affect CML-BSA-induced activities. These results suggested that CML-BSA induced γ-GCSh mainly through AP-1. Previously, Zipper and Mulcahy [41] reported that the induction of γ-GCSh gene were suppressed by inhibition of ERK and p38^{MAPK}. In the present study, CML-BSA stimulated PKC, Raf-1, and ERK1/2 activities (Fig. 4). Considered together, these data suggest that ERK is important for the AP-1-induced γ-GCSh expression under the conditions employed in our experiments.

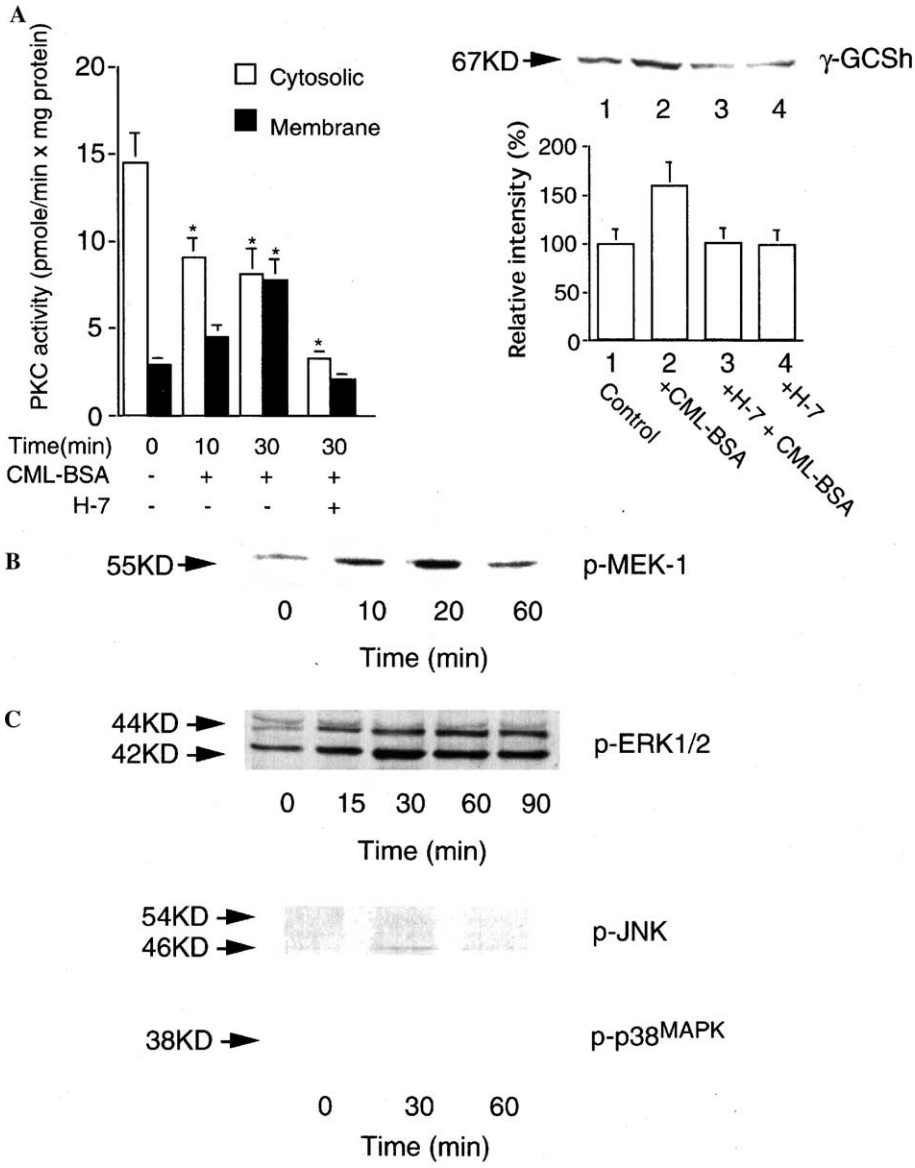

Fig. 4. Activity of PKC and Raf kinase, and MAPK assay. PKC activity (A, left), Raf kinase activity (B), phosphorylation of ERK1/2 (C, top), JNK (C, middle), and p38MAPK (C, bottom) after treatment of cells with 100 μg/ml of CML-BSA were measured at the indicated incubation times. PKC activity was measured in the cytosolic and membrane fractions of RAW264.7 cells using a PKC enzyme assay system (Amersham, Arlington Heights, IL). H-7 (a PKC inhibitor, 20 μM) was added 30 min before CML-BSA treatment. The relative intensity of γ-GCSh protein induced by CML-BSA was estimated with or without H-7 by Western blot analysis. (A, right) Activity of Raf kinase was measured using an immune complex kinase assay. Western blot analysis was employed to measure the phosphorylation of ERK1/2, JNK, and p38MAPK.

Recent studies have shown that transfection of γ-GCSh enhances GSH synthesis and suppresses TNF-α-induced activation of AP-1 [42], indicating that GSH controls the redox status of cells. On the other hand, we reported previously the lack of redox regulation in endothelial cells exposed to high concentrations of glucose over long period [17]. Impairment of redox regulation is thus thought to be present in diabetic conditions and may cause inadequate responses of cells to AGEs leading to the development of diabetic complications.

In diabetic patients, AGEs directly alter extracellular proteins through glycation-induced cross-link formation and affect the interaction between endothelial cells and the extracellular matrix, resulting in the impairment of neovascularization [43]. We have also reported recently that AGEs induce the expression of vascular endothelial growth factor (VEGF) [44]. Since up-regulation of VEGF expression is a major event leading to vascularization, the induction of VEGF by AGEs may correlate with the capacity of macrophages to promote neovascularization and enhance vascular permeability in diabetic microangiopathy [45]. It is believed that VEGF induction is important in diabetic conditions, such as retinopathy and renopathy [43]. Similarly, AGEs have

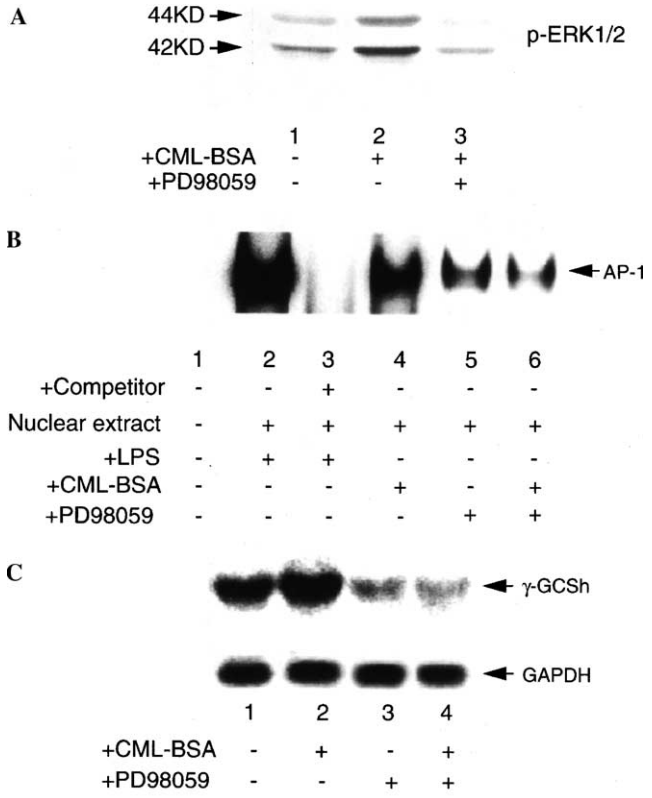

Fig. 5. Effect of ERK1/2 inhibitor. Effects of PD98059 [2-(2′-amino-3′-methylphenyl)oxanaphthalene-4-one], a specific inhibitor of MEK 2, on CML-BSA-induced AP-1-DNA binding activity and γ-GCSh mRNA expression. RAW264.7 cells were incubated with 50 μM PD98059 for 1 h then treated with 100 or 200 μg/ml of CML-BSA. (A) ERK1/2 activity on Western blots; (B) AP-1 activity on electrophoretic mobility shift assay; (C), γ-GCSh mRNA expression on Northern blots.

been reported to induce the expression of the gene for tissue factor [46] in vascular tissues, and those for monocyte chemoattractant peptide and IL-6 [47] in vascular smooth muscles. These data support the notion that AGEs play a pathological role in diabetic atherogenesis by leading proinflammatory mediators in vessel wall.

Intracellular GSH plays a role in scavenging ROS and regulating redox state. As an anti-oxidant, GSH scavenges hydrogen peroxide by the reaction of GSH peroxidase. Redox-related modifications of protein cysteine residues have emerged in recent years as the molecular mechanisms underlying many cellular processes, including signal pathways and DNA transcription [16]. GSH is involved in the regulation of redox state through enzymatic reaction by thioredoxin and glutaredoxin, or non-enzymatic modification of proteins such as thiol groups [48]. In human endothelial cells, CML-modified adducts enhance the generation of hydrogen peroxide via activation of NADPH oxidase [49]. Moreover, we have demonstrated the involvement of ROS in the induction of VEGF by AGEs in RAW264.7 cells [44]. However, in the present study, production of ROS was not observed in RAW264.7 cells treated with CML-BSA (data not shown). The reason might be due to differences in cell types, or between AGEs and CML.

In conclusion, we have demonstrated in the present study that CML-BSA induced the expression of γ-GCSh mRNA mainly by AP-1 through activation of the intracellular cascade, PKC, Raf-1, and ERK1/2, in macrophages. γ-GCS up-regulation may serve to protect the cells against inflammatory reactions and oxidative stress.

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