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N-Epsilon-(carboxymethyl)lysine is unable to induce endothelial dysfunction but is able to attenuate AGEs-induced endothelium damage in human umbilical vein endothelial cells

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Received August 29, 2012, accepted October 15, 2012

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Pharmazie 68: 251–256 (2013) *doi: 10.1691/ph.2013.2765*

N-Epsilon-carboxymethyllysine (CML), one of the main compounds in advanced glycation end products (AGEs), has been thought to be a high bioaffinity ligand of the receptor for AGEs (RAGE), and is involved in the pathogenesis in endothelial dysfunction in diabetic vascular complications. However, some researchers believed that CML was unable to bind to RAGE and could not induce endothelium damage. In our present experiment, the role of CML in inducing endothelial dysfunction, preventing AGEs-induced damage and binding to RAGE was explored in human umbilical vein endothelial cells (HUVECs). The treatment with CML could not induce the endothelial dysfunction by itself, including upregulation on transforming growth factor-β1 (TGF-β1), intercellular adhesion molecule-1 (ICAM-1) and RAGE proteins expressions, apoptosis and cell viability in HUVECs. However, pretreatment with CML could attenuate AGEs-induced endothelial dysfunction. Fluorescence polarization assay showed that CML had a bioaffinity to RAGE. The IC_{50} of CML binding to RAGE (10 ng and 100 ng) were 7.133×10^{-8}M and 1.563×10^{-6}M, respectively. Our findings indicate that CML has no cytotoxic injury to endothelium but has a bioaffinity for the binding to RAGE.

1. Introduction

N-Epsilon-(carboxymethyl)lysine (CML, Fig. 1), one of the most abundant products of advanced glycation end products (AGEs), is formed in the glycation and oxidation of protein products, and amino acids by non-enzymatic glycation reaction (Anderson et al. 1999; Kislinger et al. 1999). CML was commonly considered as a contributer for endothelial dysfunction which might lead to diabetic vascular complications (Matsumoto et al. 2010; Singh et al. 2001). Accumulating evidence shows that CML is significantly increased in patients with diabetes mellitus and has been used as biochemical marker for the onset or progression of diabetic complications (Ghanem et al. 2010; Semba et al. 2009; Franke et al. 2003). Moreover, a significant increase of CML levels was associated with the aggravation of diabetic vascular complications (Tochino et al. 2007). Studies have revealed that the underlying mechanism is to bind specifically to the receptor for AGEs (RAGE) and then trigger inflammatory response in the vascular complications (Kislinger et al. 1999). It has also been found that the accumulation of CML upregulates RAGE expression on podocytes (Tanji et al. 2000).

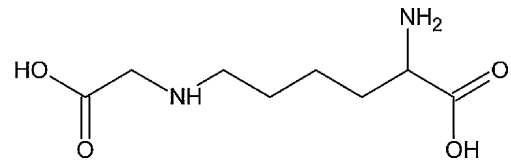


Fig. 1: Chemical structure of N-epsilon-(carboxymethyl)lysine (CML), chemical formula: $C_8H_{16}N_2O_4$, molecular weight = 204.23

However, the role of CML has been argued in the activation of pro-inflammatory pathways and the pathogenesis of vascular complications. Some researchers believed that CML might not form the necessary structure to interact with RAGE and then activate an inflammatory signaling cascade in RAGE-expressing cells (Buetler et al. 2008). Research also showed that no proinflammatory cytokine Intercellular Adhesion Molecule-1 (ICAM-1) expression was found on endothelial cells with incubation of CML (Lieuw-a-Fa et al. 2006). Valencia et al. (2004) also supported this overview, namely, CML is not sufficient to induce inflammatory signals based on the lack of activity. Accordingly, what is the role of CML in the AGEs-RAGE axis in vascular complications? Could it bind to RAGE and then activate a series of pathological signal cascade?

Therefore, the aim of the present study was to investigate the role of CML in AGEs-endothelial dysfunction including apoptosis, inflammation and RAGE expression in human umbilical vein endothelial cells (HUVECs) and explore its possibility to bind to RAGE.

Abbreviations: CML, N-epsilon-(carboxymethyl)lysine; AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end products; ICAM-1, intercellular adhesion molecule 1; TGF-β1, transforming growth factor-β1; HUVECs, human umbilical vein endothelial cells; BSA, bovine serum albumin; AO, acridine orange; EB, ethidium bromide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer solution; FBS, fetal bovine serum; OD, optical density; DAB, 3,3'-diaminobenzidine; SABC, StreptAvidin-Biotin-enzyme; SABC-AP, StreptAvidin-Biotin- enzyme-alkaline phosphatase.

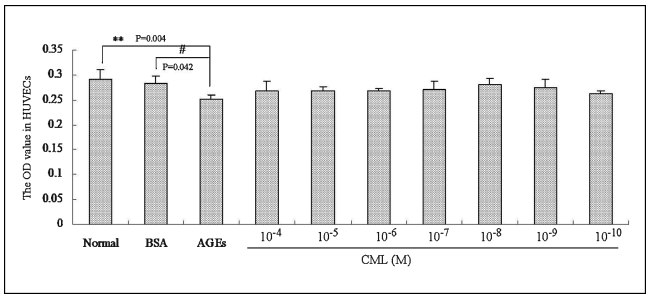


Fig. 2: Effect of CML on cell viability in HUVECs. Cells were incubated with DMEM medium, 200 μg/mL non-glycated BSA, 200 μg/mL AGEs and $10^{-10}-10^{-4}$ μM CML for 48 h. $^{**}p<0.01$, AGEs vs BSA group; $^{\#}p<0.05$ AGEs vs normal group; no significance was observed between CML and Normal or BSA. Measurements were taken in triplicate and the data were expressed as means ± standard deviation (SD)

2. Investigations and results

2.1. Effects of CML on cell viability in HUVECs

In order to examine the effect of CML on cell viability, MTT assay was performed according to the method described previously (Feng et al. 2010). As shown in Fig. 2, the OD value of AGEs group decreased significantly compared with the normal ($p=0.004$) and non-glycated BSA groups ($p=0.042$). However, no statistical difference on cell viability of CML group (from 10^{-10} to 10^{-4} μM) was observed compared with normal and BSA. Therefore, the results indicated that CML might not reduce the cell viability in HUVECs.

2.2. CML attenuated AGEs-induced apoptosis

To further demonstrate the effect of CML on endothelial dysfunction, acridine orange (AO)-ethidium bromide (EB) fluorescence staining was performed to evaluate the apoptosis of CML in HUVECs in this experiment. As depicted in Fig. 3A, the representative apoptosis characteristics including nuclear condensation, membrane blebbing and nuclear fragmentation were observed in cells which expose to 200 μg/mL AGEs ($p<0.01$, AGEs vs. BSA), whereas no apoptosis characteristics were shown in CML-treated HUVECs. Surprisingly, CML (10 μM) could not induce significantly endothelium apoptosis. Compared with AGEs, the incubation with CML alone could not induce apoposis in HUVECs ($p<0.01$). More interestingly, a significant reduction of the pretreatment with CML on apoptosis was observed as compared with AGEs or AGEs + CML ($p<0.01$, CML + AGEs vs. AGEs; CML + AGEs vs. AGEs + CML). Thus, cells in the AGEs + CML group did not show this appearance and were not significantly different, from the AGEs group. These findings demonstrated that CML could block the interaction between AGEs and HUVECs while could not induce endothelial dysfunction by itself.

2.3. CML down-regulated AGEs-induced ICAM-1 and TGF-β1 protein expression

ICAM-1, as a cell adhesion molecule, has been recognized increasingly as an important pro-inflammation factor in the initiation and the progression of vascular diseases (Sprague and Khalil 2009). In this experiment, we observed AGEs enhanced positive expression of ICAM-1. However, no obvious positive expression was observed in CML-treated HUVECs. More importantly, pretreatment with CML could significantly reduce AGEs-induced ICAM-1 expression ($p<0.01$, CML + AGEs vs AGEs group, Fig. 4B). Furthermore, there is a significant

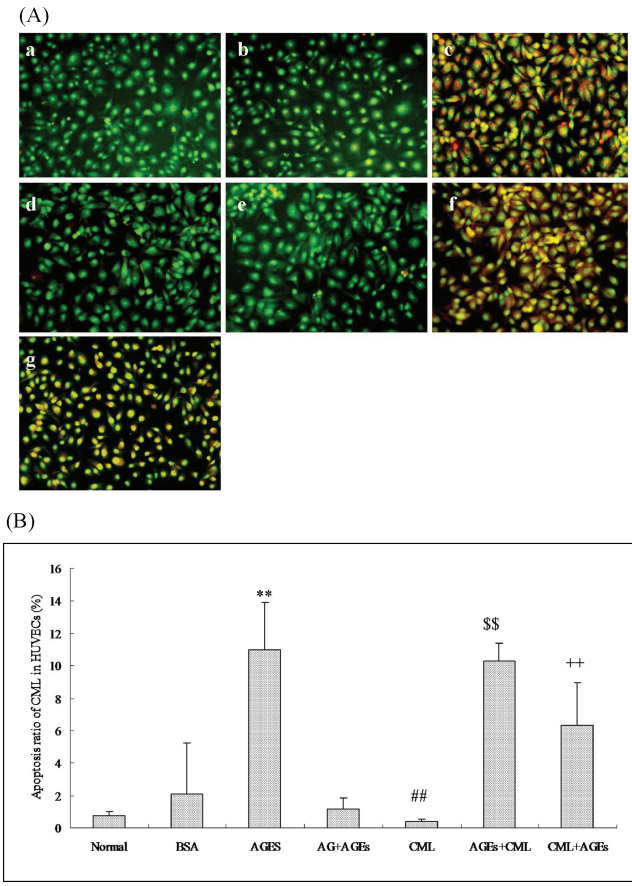


Fig. 3: Effect of CML on the apoptosis in HUVECs by AO/EB fluorescence staining. (A) AO/EB fluorescence staining. Cells were incubated with DMEM medium (Aa), 200 μg/mL non-glycated BSA (Ab), 200 μg/mL AGEs(Ac), 10 μM aminoguanidine (AG) + 200 μg/mL AGEs (Ad), 10 μM CML (Ae), 200 μg/mL AGEs + 10 μM CML (Af) and 10 μM CML + 200 μg/mL AGEs (Ag) for 48 h. And then cells were stained with 5 μL AO/EB and observed under fluorescence microscopy. Brown deposits represent positive expression (purple arrow). (B) The relative apoptosis ratio. $^{**}p<0.01$, AGEs vs BSA; $^{\#\#}p<0.01$, CML vs AGEs; $^{\$\$}p<0.01$, AGEs + CML vs BSA; $^{++}p<0.05$, CML + AGEs vs AGEs + CML. Bars in all graphs represent means ± SD of three individual experiments per group. Magnification: 200 ×

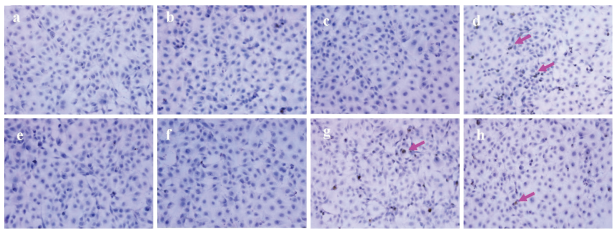
statistical difference between CML + AGEs and AGEs + CML ($p<0.01$, Fig. 4B). The results indicated that CML was not sufficient to upregulate ICAM-1 expression whereas could prevent the induction of AGEs.

TGF-β1, a cytokine associated with inflammation response, plays a pivotal role in the initiation and the aggravation of diabetic vascular complications (Gerhardinger et al. 2009; Fu et al. 2008). Herein, we observed AGEs enhanced the positive expression of TGF-β1 ($p<0.01$, vs. normal and BSA, Fig. 5A). However, CML of 10 μM did not upregulate significantly TGF-β1 positive expression as that of AGEs. Furthermore, pre-treatment of CML could attenuate AGEs-induced TGF-β1 protein expression ($p<0.01$, vs AGEs group, Fig. 5B). Interestingly, there was a significant difference between AGEs + CML and CML + AGEs ($p<0.01$). The results also supported the assumption that CML was unable to induce endothelial dysfunction in HUVECs, but could prevent the damage of AGEs.

2.4. CML reversed the effect of AGEs on RAGE expression

The accumulation and stimulation of AGEs could up-regulate RAGE protein expression and then elicit a series of signal transduction cascaded (Goldin et al. 2006). As depicted in Fig. 6A,

(A)



(B)

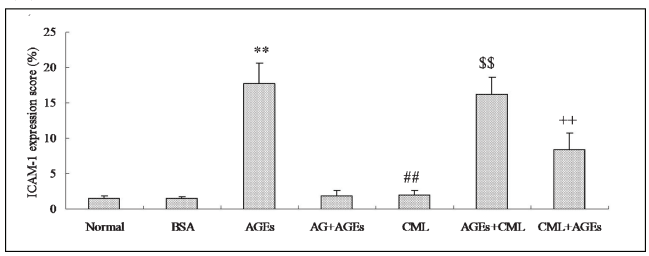
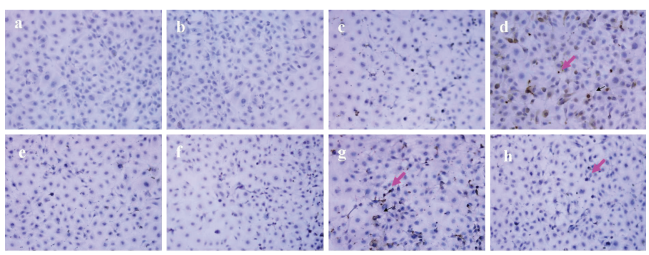


Fig. 4: Effect of CML on ICAM-1 expression in HUVECs by immunocytochemistry assay. (A) Immunocytochemistry images of ICAM-1 protein expression. The treatment of agent was same as the drug treatment of apoptosis. Primary antibody was replaced by PBS for negative control (Aa). In addition, cells were treated with DMEM medium (Ab), non-glycated BSA (Ac), AGEs (Ad), AG + AGEs (Ae), CML (Af), AGEs + CML (Ag) and CML + AGEs (Ah). (B) The relative ICAM-1 protein expression (%). $^{**}p < 0.01$, AGEs vs BSA; $^{\#\#}p < 0.01$, CML vs AGEs; $^{\$\$}p < 0.01$, AGEs + CML vs BSA; $^{++}p < 0.05$, CML + AGEs vs AGEs + CML. Bars in all graphs represent means ± SD of three individual experiments per group. Purple arrows represent positive expression. Magnification: 200×

(A)



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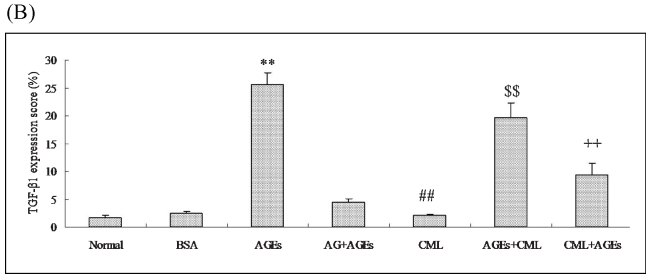
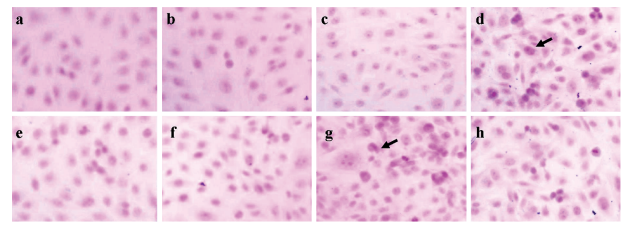


Fig. 5: Effect of CML on TGF-β1 expression in HUVECs by immunocytochemistry assay. (A) Immunocytochemistry images of TGF-β1 protein expression. The treatment of agent as described previously. Primary antibody was replaced by PBS for negative control (Aa). Additionally, cells were treated with DMEM medium (Ab), non-glycated BSA (Ac), AGEs (Ad), AG + AGEs (Ae), CML (Af), AGEs + CML (Ag) and CML + AGEs (Ah). (B) The relative TGF-β1 protein expression (%). $^{**}p < 0.01$, AGEs vs BSA; $^{\#\#}p < 0.01$, CML vs AGEs; $^{\$\$}p < 0.01$, AGEs + CML vs BSA; $^{++}p < 0.05$, CML + AGEs vs AGEs + CML. Bars in all graphs represent means ± SD of three individual experiments per group. Purple arrows represent positive expression. Magnification: 200 ×

AGEs up-regulated the expression of RAGE whereas CML did not show this ability. Moreover, the pre-incubation with CML (10 μM) might attenuate AGEs-induced RAGE protein expression as compared with post-incubation of CML ($p < 0.01$, Fig. 6B). All these findings suggested that CML was not sufficient to upregulate RAGE protein expression whereas could attenuate that of AGEs-motivated.

(A)



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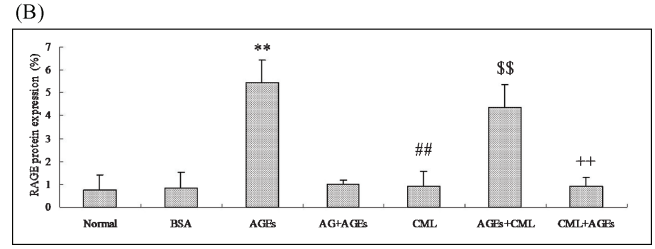

Fig. 6: Effect of CML on RAGE expression in HUVECs by immunocytochemistry assay. (A) RAGE protein expression. Primary antibody was replaced by PBS for negative control (Aa). And then cells were treated with DMEM medium (Ab), non-glycated BSA (Ac), AGEs (Ad), AG + AGEs (Ae), CML (Af), AGEs + CML (Ag) and CML + AGEs (Ah). (B) The relative RAGE protein expression (%). $^{**}p < 0.01$, AGEs vs BSA; $^{\#\#}p < 0.01$, CML vs AGEs; $^{\$\$}p < 0.01$, AGEs + CML vs BSA; $^{++}p < 0.05$, CML + AGEs vs AGEs + CML. Bars in all graphs represent means ± SD of three individual experiments per group. Black arrow represents positive expression. Magnification: 400 ×

2.5. *Binding property of CML to RAGE by fluorescence polarization*

In order to evaluate the binding ability of CML to RAGE, a fluorescence polarization method was used for the determination of the polarization of RAGE in this experiment. As shown in Fig. 7, an increasing on the polarization of RAGE (10 ng and 100 ng) was observed with the increasing concentration of CML (form 10^{-12} M to 10^{-4}M). The IC_{50} of CML binding to RAGE (10 ng and 100 ng) were $IC_{50} = 7.133 \times 10^{-8}$M and $IC_{50} = 1.563 \times 10^{-6}$M, respectively. The results indicated that CML has a significant binding ability to RAGE.

3. Discussion

The accumulation of CML has been implicated in the pathogenesis of vasculopathy in diabetic vascular complications (Vay et al. 2000). There is a growing body of evidence that CML is a ligand that has high affinity for binding to RAGE (Penfold et al. 2010; van Deutekom et al. 2008). However, the opposing view is that CML is unable to bind to RAGE and cannot activate a pathological response (Buetler et al. 2008). In this study, we provided confirmatory evidence to demonstrate that

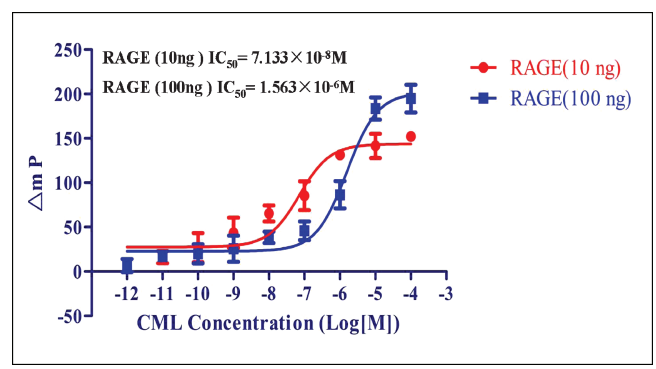

Fig. 7: Binding curves of CML and RAGE proteins by fluorescence polarization. RAGE of 10 ng or 100 ng was incubated for 4 h in absence or presence of CML (10^{-12} to 10^{-4} M). Fluorescence polarization of sample was determined at excitation/emission wavelengths of 295 nm/350 nm

CML could bind to RAGE but was not able to induce endothelial dysfunction. Moreover, pretreatment with CML might attenuate AGEs-induced cell damage in HUVECs under our experimental conditions.

AGEs was found to reduce cell viability (Bigl et al. 2007). Several reports also demonstrated that CML contributed to extracellular matrix dysfunction and led to the decreasing of cell viability (Semba et al. 2009; Franke et al. 2003). Although the argumentation on whether CML had cytotoxic effect in cells was present, we found that AGEs could reduce the cell viability of HUVECs, whereas CML did not show cytotoxicity in HUVECs at a concentration of 10^{-10}–10^{-4} g/mL. These results indicated that CML might not be a risk factor for endothelial dysfunction in diabetic vascular complications.

The effect of CML on cell viability in HUVECs may be correlated with many possible mechanisms, such as apoptosis response (Fu et al. 1996). Apoptosis was also regarded as one of underlying mechanisms on cellular damage contributing to diabetic vascular complications via activating RAGE (Gasic-Milenkovic et al. 2001). In previous studies, CML has been shown to induce apoptosis and reduce cell proliferation (Lee et al. 2010). However, CML could not induce the typical apoptosis in HUVECs as compared with normal and BSA groups under our experimental conditions (Fig. 3). Interestingly, CML could significantly reduce the apoptosis degree of AGEs. It may be related with the blockade of CML on interaction of AGEs with its receptor.

Inflammation response plays a crucial role in AGEs-induced endothelial dysfunction (Kneyber et al. 2009). ICAM-1, a characteristical marker of the development of diabetic vascular complications, is responsible for inflammation response (Boulanger et al. 2004). It has been shown that CML-modified adducts also could enhance the levels of mRNA and antigen for ICAM-1 (Matsui et al. 1996). However, Sjögren et al. (2007) found that CML was not a risk factor for endothelial activation and didn't increase the level of ICAM-1. In our present experiment, CML could not significantly increase ICAM-1 expression in HUVECs as AGEs (Fig. 5A). Additionally, ICAM-1 expression stimulated by AGEs was significantly decreased after being pre-treated with CML. All of these results were in agreement with the report of Lieuw-a-Fa et al. (2006).

TGF-β1 is regarded as an important factor in the development of diabetic vascular complications. Studies have shown that the accumulation of AGEs can up-regulate TGF-β1 protein expression and promote the pathogensis of endothelial dysfunction (Aronson 2002). However, the positive protein expression of TGF-β1 in HUVECs after being stimulated by CML was not observed in our experiment (Fig. 6). Additionally, the AGEs-induced TGF-β1 protein expression could also be attenuated significantly by the pretreatment with CML. These results indicated that CML could not induce TGF-β1 protein expression but rather prevent the interaction between AGEs and its receptor.

RAGE is a single-transmembrane and multiligand member receptor. Recent attention has been focused on the interaction of RAGE with AGEs and CML in that they activate signal transduction for diabetic complications (Collison et al. 2002). The activation of RAGE can induce oxidative stress, apoptosis and inflammation responses. It has been shown that ligands of RAGE can induce sustained activation of RAGE and upregulate RAGE expression on the surface of the cell membrane as a result of endogenous negative feedback mechanisms (Bierhaus et al. 2001). However, this upregulation of CML on RAGE protein expression was not observed under our experimental conditions. It might be associated with the insignificance of mediating signal transduction in that the structure of CML.

Why CML can attenuate AGEs-induced apoptosis, ICAM-1, TGF-β1 and RAGE protein expressions in HUVECs, but not by itself? There are two possibilities for this phenomenon: on one hand, CML may competitively bind to RAGE with AGEs and block AGEs-RAGE interaction. Thus, being pretreated with cells, CML may have priority to bind to receptor to prevent the binding of toxic AGEs to RAGE. On the other hand, CML cannot induce endothelial dysfunction, including apoptosis and proinflammatory responses, according to its chemical structure. It has been well shown that CML modification, such as the glycation proteins containing CML, alters its biological properties for mediating pathological signaling cascades in that its larger chemical structure (Yoshinaga et al. 2011). However, non-modified CML, not containing glycation proteins, is a small molecule compound. The pathological signaling required for endothelial dysfunction might not be generated even if the binding of CML to RAGE because of its small structure.

Previous reports indicated that CML could bind to RAGE and lead to diabetic complications and inflammatory disorders (Southern et al. 2007; Anderson et al. 1999). However, Buetler et al. (2008) proposed a contrary view that CML-modified bLG or HAS were unable to bind to RAGE in a cell-free assay system. Our results showed that incubation of CML with RAGE increased mP at a concentration of 10^{-4}–10^{-12} g/mL (Fig. 7). Combined with the above results, we believe that CML had a bioaffinity for binding to RAGE on the surface of the cell membrane in HUVECs.

AGEs are also highly specific binding ligands for RAGE (Nienhuis et al. 2009). The structure of argpyrimidine, pyrraline, CML, OP-lysine, glyoxal–lysine dimer, pentosidine and glucosepane in AGEs all have common carbonyl groups which may be responsible for the binding to RAGE (Xu et al. 2010). The structure of CML contains two negatively charged carboxyl groups at both ends of the chemical structure determining the polarity of the carbonyl group and the binding to RAGE. Recently, accumulating evidence showed that RAGE was a pattern recognition receptor. Namely, all ligands contain one or more net negative charge tail responsible for the recognition of RAGE (Fritz 2011). For a RAGE ligand HMGB1, its COOH-terminal motif was responsible for the binding to RAGE (Huttunen et al. 2002). Taken together, these findings strongly support our view that CML could bind to RAGE. However, more experiments need to be performed further to prove this view.

In conclusion, the present results suggested that CML could not induce the endothelial dysfunction in HUVECs, but could attenuate the AGEs-induced endothelium damage. Our study also supported that CML has a bioaffinity for binding to RAGE. However, this property may be innocuous for the pathogenesis of diabetic complications.

4. Experimental

4.1. Materials

Receptor of advanced glycation end products peptide (RAGE) was obtained from Santa Cruz Biotechnology, Inc. (USA). N-Epsilon-(carboxymethyl)lysine (CML) was ordered from Toronto Research Chemicals Inc. (North York, Canada). Bovine serum albumin (BSA), D-glucose, acridine orange (AO), ethidium bromide (EB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). StreptAvidin-Biotin-enzyme (SABC); StreptAvidin-Biotin-enzyme-alkaline phosphatase (SABC-AP) were obtained from Boster Biological Engineering Co., Ltd. (Wuhan, P.R. China). All other materials were analytical reagent grade and from commercial sources.

4.2. AGEs preparation

AGEs was prepared as described previously with minor modifications (Takeuchi et al. 2000; Sheikpranbabu et al. 2010). BSA of 5 g was incubated in 100 ml phosphate buffer (0.2 M, pH 7.4) under sterile conditions with D-glucose of 9 g for 12 weeks. At the end of the incubation period, unincorporated sugars were removed by dialysis overnight against phosphate-buffered

saline. In addition, non-glycated BSA was prepared in the same conditions for 12 weeks with the exception of the absence of reducing sugars for blank control. The obtained AGEs and non-glycated BSA were determined for the content control at excitation/emission wavelengths of 370 nm/440 nm. Finally, AGEs and non-glycated BSA were stored at 4 °C for further experiments.

4.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) line was obtained from ATCC (Manassas, VA, USA). Cells were maintained in low-glucose DMEM medium supplemented with 5% fetal bovine serum, 100 units/mL of penicillin/streptomycin according to the manufacturer's instructions (Clonetics Corp., San Diego, CA). Cells were maintained in 5% CO_2 at 37 °C and the medium was renewed every 2 days. After 80–90% confluent, cells were used for the further experiments.

4.4. MTT assay for cell viability

HUVECs was seeded into 96-well plates with 100 μL/well and incubated at 37 °C and 5% CO_2 for 24 h. After being starved for 12 h, cells were treated with agents for 48 h. The cells were treated with final concentration 5 mg/mL MTT and maintained at 37 °C for 4 h. Formazan was dissolved with DMSO of 100 μL. The optical density (OD) value in wells was read at 450 nm with a microplate reader.

4.5. Fluorescence detection with acridine orange-ethidium bromide staining

In order to observe the effect of CML on apoptosis, HUVEC cells were stained with acridine orange/ethidium bromide (AO/EB) according to method as previously described (Li et al. 2010). In brief, cells were exposed to DMEM medium, non-glycated BSA (200 μg/mL), CML (10 μM), AGEs (200 μg/mL), CML (10 μM) + AGEs (200 μg/mL) and AGEs (200 μg/mL) + CML (10 μM). The concentration of CML was chosen in this study as previously described (Buetler et al. 2008). Aminoguanidine (AG) of 10 μM was used for the positive control. After incubation for 48 h, cells were treated with 5 μL prepared AO/EB solution containing 100 μg/mL AO and 100 μg/mL EB in PBS and then observed under a fluorescence microscope (Olympus IX71 inversion microscope, Japan). The difference in cells was calculated according to green (normal cells) or jacinth (apoptosis cells) color area with Image-Pro Plus picture analysis software.

4.6. Immunocytochemistry staining for ICAM-1, TGF-β1 and RAGE expression

Immunocytochemistry staining was performed as described previously for the evaluation of ICAM-1, TGF-β1 and RAGE expression (Xu et al. 2010). In brief, HUVECs were seeded on glass coverslips in 24-well plates. After being incubated with agents, the cells were washed with PBS and fixed with fresh 4% paraformaldehyde for 90 min. Then 10% primary antibody-origin serum or 5% BSA (for RAGE detection) was used to block the cells for 20 min at room temperature. The blocked cells were incubated with ICAM-1 (1:200), TGF-β1 (1:400) and RAGE (1:200) antibodies at 37 °C, respectively. After being incubated for 20 min, cells were incubated with biotin-conjugated secondary antibody and StreptAvidin-Biotin-enzyme (SABC) or StreptAvidin-Biotin-enzyme-alkaline phosphatase (SABC-AP) at 37 °C. 3,3'-Diaminobenzidine (DAB) or BCIP/NBT (for RAGE) was used for colarization. Finally, the coverslips were counterstained with hematoxylin or nuclear fast red (for RAGE) and observed under microscope.

4.7. Fluorescence polarization assay

Samples were conducted in black 96-well plates (Corning, Inc., USA) and fluorescence polarization assay was performed in a darkdrawer of LJL biosystems (Analyst^{TM} AD, Sunnyvale, CA, USA). RAGE was incubated with CML for 4 h at 37 °C. Fluorescence polarization of RAGE (10 ng and 100 ng) in the presence or absence of CML (10^{-12} to 10^{-4} M) was determined at excitation/emission wavelength of 295 nm/350 nm according to the punlished detection wavelength of RAGE (Gospodarska et al. 2011).

4.8. Data analysis

Fluorescence polarization data were expressed as millipolarization units (mP). The total change of polarization, which represents bound fraction of CML to RAGE, was calculated according to the following equation as previously described:

$$P = P_{max} - P_{min}$$

where P_{max} is measured polarization of samples, and P_{min} is the polarization of RAGE in the absence of CML. In all analyses, statistical differences were evaluated by one-way ANOVA with SPSS 16.0 software. The level of significance was set at $p < 0.05$.

Acknowledgments: This work was supported by grants from National Natural Science of China (No. 81202906) and Natural Science Foundation of Jiangsu Province (BK2012491).

References

Anderson MM, Requena JR, Crowley JR, Thorpe SR, Heinecke JW (1999) The myeloperoxidase system of human phagocytes generates Nepsilon-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. J Clin Invest 104: 103–113.

Aronson D (2002) Potential role of advanced glycosylation end products in promoting restenosis in diabetes and renal failure. Med Hypotheses 59: 297–301.

Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, Kislinger T, Stern DM, Schmidt AM, De Caterina R (2002) Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 105: 816–822.

Bierhaus A, Schiekofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J, Hong M, Luther T, Henle T, Klöting I, Morcos M, Hofmann M, Tritschler H, Weigle B, Kasper M, Smith M, Perry G, Schmidt AM, Stern DM, Häring HU, Schleicher E, Nawroth PP (2001) Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes 50: 2792–2808.

Bigl K, Schmitt A, Meiners I, Münch G, Arendt T (2007) Comparison of results of the CellTiter Blue, the tetrazolium (3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyl tetrazolium bromide), and the lactate dehydrogenase assay applied in brain cells after exposure to advanced glycation endproducts. Toxicol *In Vitro* 21: 962–971.

Boulanger E, Wautier MP, Gane P, Mariette C, Devuyst O, Wautier JL (2004) The triggering of human peritoneal mesothelial cell apoptosis and oncosis by glucose and glycoxydation products. Nephrol Dial Transplant 19: 2208–2216.

Buetler TM, Leclerc E, Baumeyer A, Latado H, Newell J, Adolfsson O, Parisod V, Richoz J, Maurer S, Foata F, Piguet D, Junod S, Heizmann CW, Delatour T (2008) N(epsilon)-carboxymethyllysine-modified proteins are unable to bind to RAGE and activate an inflammatory response. Mol Nutr Food Res 52: 370–378.

Collison KS, Parhar RS, Saleh SS, Meyer BF, Kwaasi AA, Hammami MM, Schmidt AM, Stern DM, Al-Mohanna FA (2002) RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). J Leukoc Biol 71: 433–444.

Feng L, Jia XB, Jiang J, Zhu MM, Chen Y, Tan XB, Shi F (2010) Combination of active components enhances the efficacy of Prunella in prevention and treatment of lung cancer. Molecules 15: 7893–7906.

Franke S, Dawczynski J, Strobel J, Niwa T, Stahl P, Stein G (2003) Increased levels of advanced glycation end products in human cataractous lenses. J Cataract Refract Surg 29: 998–1004.

Fritz G (2011) RAGE: a single receptor fits multiple ligands. Trends Biochem Sci 36: 625–632.

Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR (1996) The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. J Biol Chem 271: 9982–9986.

Fu K, Corbley MJ, Sun L, Friedman JE, Shan F, Papadatos JL, Costa D, Lutterodt F, Sweigard H, Bowes S, Choi M, Boriack-Sjodin PA, Arduini RM, Sun D, Newman MN, Zhang X, Mead JN, Chuaqui CE, Cheung HK, Zhang X, Cornebise M, Carter MB, Josiah S, Singh J, Lee WC, Gill A, Ling LE (2008) SM16, an orally active TGF-beta type I receptor inhibitor prevents myofibroblast induction and vascular fibrosis in the rat carotid injury model. Arterioscler Thromb Vasc Biol 28: 665–671.

Gasic-Milenkovic J, Loske C, Deuther-Conrad W, Münch G (2001) Protein "AGEing"–cytotoxicity of a glycated protein increases with its degree of AGE-modification. Z Gerontol Geriatr 34: 457–460.

Gerhardinger C, Dagher Z, Sebastiani P, Park YS, Lorenzi M (2009) The transforming growth factor-beta pathway is a common target of drugs that prevent experimental diabetic retinopathy. Diabetes 58: 1659–1667.

Ghanem AA, Elewa A, Arafa LF (2011) Pentosidine and N-carboxymethyllysine: biomarkers for type 2 diabetic retinopathy. Eur J Ophthalmol 21: 48–54.

Goldin A, Beckman JA, Schmidt AM, Creager MA (2006) Advanced glycation end products: Sparking the development of diabetic vascular injury. Circulation 14: 597–605.

Gospodarska E, Kupniewska-Kozak A, Goch G, Dadlez M. 2011. Binding studies of truncated variants of the Aβ peptide to the V-domain of the RAGE receptor reveal Aβ residues responsible for binding. Biochim Biophys Acta 1814: 592–609.

Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H (2002) Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. Cancer Res 62: 4805–4811.

Kislinger T, Fu C, Huber B, Qu W, Taguchi A, Du Yan S, Hofmann M, Yan SF, Pischetsrieder M, Stern D, Schmidt AM (1999) 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. J Biol Chem Oct 274: 31740–31749.

Kneyber MC, Gazendam RP, Niessen HW, Kuiper JW, Dos Santos CC, Slutsky AS, Plötz FB (2009) Mechanical ventilation during experimental sepsis increases deposition of advanced glycation end products and myocardial inflammation. Crit Care 13: R87.

Lee BW, Chae HY, Kwon SJ, Park SY, Ihm J, Ihm SH (2010) RAGE ligands induce apoptotic cell death of pancreatic β-cells via oxidative stress. Int J Mol Med 26: 813–818.

Li Y, Wang MY, Fan XS, Qi X, Chen Y, Zhang H, Zhao QC (2010) Effect of San-ao Decoction, a traditional Chinese prescription, on IL-4 treated normal human bronchial epithelium. J Ethnopharmacol 131: 104–109.

Lieuw-a-Fa ML, Schalkwijk CG, Engelse M, van Hinsbergh VW (2006) Interaction of Nepsilon(carboxymethyl)lysine- and methylglyoxal-modified albumin with endothelial cells and macrophages. Splice variants of RAGE may limit the responsiveness of human endothelial cells to AGEs. Thromb Haemost 95: 320–328.

Matsui H, Suzuki M, Tsukuda R, Iida K, Miyasaka M, Ikeda H (1996) Expression of ICAM-1 on glomeruli is associated with progression of diabetic nephropathy in a genetically obese diabetic rat, Wistar fatty. Diabetes Res Clin Pract 32: 1–9.

Matsumoto T, Ozawa Y, Taguchi K, Kobayashi T, Kamata K (2010) Diabetes-associated changes and role of Nepsilon-(carboxymethyl)lysine in big ET-1-induced coronary vasoconstriction. Peptides 31: 346–353.

Nienhuis HL, Westra J, Smit AJ, Limburg PC, Kallenberg CG, Bijl M (2009) AGE and their receptor RAGE in systemic autoimmune diseases: an inflammation propagating factor contributing to accelerated atherosclerosis. Autoimmunity 42: 302–304.

Penfold SA, Coughlan MT, Patel SK, Srivastava PM, Sourris KC, Steer D, Webster DE, Thomas MC, MacIsaac RJ, Jerums G, Burrell LM, Cooper ME, Forbes JM (2010) Circulating high-molecular-weight RAGE ligands activate pathways implicated in the development of diabetic nephropathy. Kidney Int 78: 287–295.

Semba RD, Bandinelli S, Sun K, Guralnik JM, Ferrucci L (2009) Plasma carboxymethyl-lysine, an advanced glycation end product, and all-cause and cardiovascular disease mortality in older community-dwelling adults. J Am Geriatr Soc 57: 1874–1880.

Sheikpranbabu S, Haribalaganesh R, Lee KJ, Gurunathan S (2010) Pigment epithelium-derived factor inhibits advanced glycation end products-induced retinal vascular permeability. Biochimie 92: 1040–1051.

Singh R, Barden A, Mori T, Beilin L (2001) Advanced glycation end products: a review. Diabetologia 44: 129–146.

Sjögren P, Basta G, de Caterina R, Rosell M, Basu S, Silveira A, de Faire U, Vessby B, Hamsten A, Hellenius ML, Fisher RM (2007) Markers of endothelial activity are related to components of the metabolic syndrome, but not to circulating concentrations of the advanced glycation end-product N epsilon-carboxymethyl-lysine in healthy Swedish men. Atherosclerosis 195(2): e168–e175.

Southern L, Williams J, Esiri MM (2007) Immunohistochemical study of Nepsilon- carboxymethyl lysine (CML) in human brain: relation to vascular dementia. BMC Neurol 16: 1–8.

Sprague AH, Khalil RA (2009) Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem. Pharmacol 78: 539–552.

Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD (2000) Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. J Am Soc Nephrol 11: 1656–1666.

Takeuchi M, Bucala R, Suzuki T, Ohkubo T, Yamazaki M, Koike T, Kameda Y, Makita Z (2000) Neurotoxicity of advanced glycation end-products for cultured cortical neurons. J Neuropathol Exp Neurol 59: 1094–1105.

Tochino Y, Kanazawa H, Ichimaru Y, Asai K, Kyoh S, Hirata K (2007) Nepsilon-(carboxymethyl)lysine, a major advanced glycation end product in exhaled breath condensate as a biomarker of small airway involvement in asthma. J Asthma 44: 861–866.

Valencia JV, Mone M, Koehne C, Rediske J, Hughes TE (2004) Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products. Diabetologia 47: 844–852.

van Deutekom AW, Niessen HW, Schalkwijk CG, Heine RJ, Simsek S (2008) Increased Nepsilon-(carboxymethyl)-lysine levels in cerebral blood vessels of diabetic patients and in a (streptozotocin-treated) rat model of diabetes mellitus. Eur J Endocrinol 158: 655–660.

Vay D, Vidali M, Allochis G, Cusaro C, Rolla R, Mottaran E, Bellomo G, Albano E (2000) Antibodies against advanced glycation end product Nepsilon-(carboxymethyl)lysine in healthy controls and diabetic patients. Diabetologia 43: 1385–1388.

Xu Y, Toure F, Qu W, Lin L, Song F, Shen X, Rosario R, Garcia J, Schmidt AM, Yan SF (2010) Advanced glycation end product (AGE)-receptor for AGE (RAGE) signaling and up-regulation of Egr-1 in hypoxic macrophages. J Biol Chem 285: 23233–23240.

Xu Y, Wang S, Feng L, Zhu Q, Xiang P, He B (2010) Blockade of PKC-beta protects HUVEC from advanced glycation end products induced inflammation. Int Immunopharmacol 10: 1552–1559.

Yoshinaga E, Kawada A, Ono K, Fujimoto E, Wachi H, Harumiya S, Nagai R, Tajima S (2011) N(ε)-(carboxymethyl)lysine modification of elastin alters its biological properties: implications for the accumulation of abnormal elastic fibers in actinic elastosis. J Invest Dermatol 132: 315–323.