DOI 10.1002/mnfr.200700101 Mol. Nutr. Food Res. 2008, *52*, 370–378

Research Article

N$^{\varepsilon}$-carboxymethyllysine-modified proteins are unable to bind to RAGE and activate an inflammatory response

Timo M. Buetler[1], Estelle Leclerc[2], Alexandra Baumeyer[1], Helia Latado[1], John Newell[1], Oskar Adolfsson[1], Véronique Parisod[1], Janique Richoz[1], Sarah Maurer[1], Francis Foata[1], Dominique Piguet[1], Sylviane Junod[1,*], Claus W. Heizmann[3] and Thierry Delatour[1a]

[1] Nestlé Research Center, Lausanne, Switzerland
[2] Florida Atlantic University, Department of Chemistry and Biochemistry, Boca Raton, FL, USA
[3] University Children's Hospital, Division of Clinical Chemistry and Biochemistry, Zürich, Switzerland

Advanced glycation endproducts (AGEs) containing carboxymethyllysine (CML) modifications are generally thought to be ligands of the receptor for AGEs, RAGEs. It has been argued that this results in the activation of pro-inflammatory pathways and diseases. However, it has not been shown conclusively that a CML-modified protein can interact directly with RAGE. Here, we have analyzed whether beta-lactoglobulin (bLG) or human serum albumin (HSA) modified chemically to contain only CML (10–40% lysine modification) can (i) interact with RAGE *in vitro* and (ii) interact with and activate RAGE in lung epithelial cells. Our results show that CML-modified bLG or HSA are unable to bind to RAGE in a cell-free assay system (Biacore). Furthermore, they are unable to activate pro-inflammatory signaling in the cellular system. Thus, CML probably does not form the necessary structure(s) to interact with RAGE and activate an inflammatory signaling cascade in RAGE-expressing cells.

Keywords: Carboxymethyllysine / Inflammation / Lactoglobulin / Maillard reaction / RAGE

Received: March 13, 2007; revised: August 10, 2007; accepted: October 3, 2007

1 Introduction

Advanced glycation endproducts (AGEs) are covalent protein modifications generated primarily by the Maillard reaction. The Maillard reaction refers to the condensation reaction of a reducing sugar with the epsilon amino group of lysine residues. After a re-arrangement, this results in the formation of the Amadori product. Upon further incubation or heating, Amadori products can fragment to form reactive dicarbonyl intermediates that react further to ultimately form AGEs [1, 2]. Because reactive dicarbonyls can also be formed by other reactions such as during glycolysis [3], lipid peroxidation [4] or ascorbate oxidation [5], certain AGEs are not exclusively formed via the Maillard reaction. For protein modifications generated from reactive aldehyde intermediates of lipid peroxidation the term advanced lipoxidation endproducts (ALEs) is being used. Furthermore, AGE/ALE formation is favored in the presence of free radicals or redox-active metals ions, resulting in higher AGE/ALE levels under conditions of oxidative stress. Both AGEs and ALEs are formed endogenously but can also be absorbed from heated food.

N$^{\varepsilon}$-carboxymethyllysine (CML) is often considered the major AGE because it can be found at relatively high levels both *in vivo* and in food products. However, CML is a good example of a modification generated by multiple pathways including lipid peroxidation [4, 6] and is, thus, not exclusively representative of the Maillard reaction. Several publications report on the simultaneous presence of both CML and the lipid peroxidation adducts 4-hydroxynonenal (HNE) and malondialdehyde (MDA) at sites of inflammation [7, 8]. Furthermore, Figarola *et al.* [9] reported that streptozotocin (STZ)-induced diabetic rats treated with either of two anti-AGE/ALE drugs (LR-7, LR-74) showed reduced albuminuria, hyperlipidemia, lipid peroxidation and CML levels but this was not associated with an effect on hyperglycemia measured as plasma glucose and $HbA1_C$. These results suggest that these drugs can reduce lipid per-

Correspondence: Dr. Timo Buetler, Nestle Research Center, Vers-chez-les-Blanc, PO Box 44, 1000 Lausanne 26, Switzerland
E-mail: Timo.Buetler@rdls.nestle.com
Fax: +41-21-785-8553

Abbreviations: **AGE**, advanced glycation endproduct; **ALE**, advanced lipoxidation endproduct; **bLG**, beta-lactoglobulin; **CML**, N$^{\varepsilon}$-carboxymethyllysine; **RAGE**, receptor for AGE

* This article is dedicated to Sylviane Junod who died January 15, 2007.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

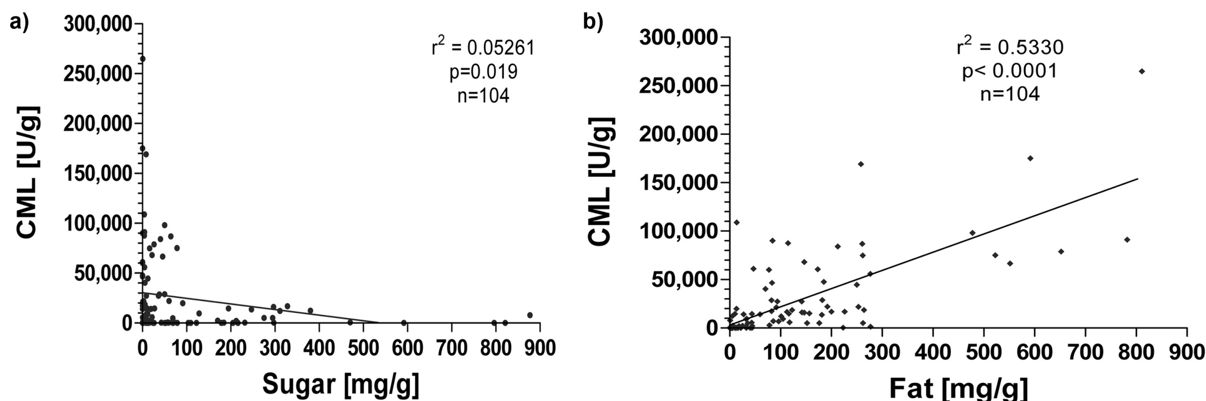

Figure 1. Correlation of CML content with sugar (A) and fat (B) content of 104 food items. CML data are from [10], the food sugar and fat content are from the Food and Nutrient Database for Dietary Studies 1.0 (http://www.ars.usda.gov/Services/docs.htm?docid=7783).

oxidation, resulting in the reduction of CML levels but not in the reduction of hyperglycemia or the glycation product of hemoglobin. Thus, effects on hyperglycemia ($HbA1_C$) can be separated from effects on hyperlipidemia/lipid peroxidation and CML appears to be associated with lipid peroxidation/hyperlipidemia rather than with hyperglycemia. In addition to endogenous formation, Goldberg *et al.* [10]. and others showed that CML is also present in heat-processed food. In this study, CML was determined in 250 food items by an indirect ELISA method. If CML was generated by the Maillard reaction, one could expect that CML would correlate with the sugar content of the food. However, a closer analysis of the Goldberg data [10] correlating the CML content with the food content of sugar and fat of some of the food items shows that CML levels do not correlate with the sugar but rather with the fat content (Fig. 1). Thus, this suggests that also in food, CML is more closely associated with fat content and probably lipid peroxidation upon heating than with the Maillard reaction.

There are few data on CML absorption from food. A study by Bergmann *et al.* [11] showed that intravenous administration of a [^{18}F]fluorobenzoyl derivative of CML to rats resulted in a rapid and complete renal elimination of circulating CML. On the other hand, in a feeding study in healthy rats Ahmed *et al.* [12] could only recover 2% of a single CML dose in the 24-h urine. In contrast, a recent study by Somoza *et al.* [13] reported a recovery of up to 29% CML in urine in rats fed a CML-enriched diet for 10 days. The high CML diet containing almost 10 g CML/kg diet resulted in an exposure of approximately 350 mg CML/kg body weight (bw)/day. In these experiments, 51% of the ingested CML were recovered of which 29% in urine and 22% in feces, the rest of the ingested CML was unaccounted for. The exposure to CML for 10 days may have resulted in a certain accumulation thus overall increasing the CML portion excreted in urine.

In her thesis work, Foerster [14] analyzed the recovery of CML in urine of human volunteers after a 1-week washout period on a low-AGE diet and after intake of a single food with high CML content (Pretzel sticks). The washout of Maillard reaction products (MRP) resulted in a clear decrease in the Amadori product fructosyllysine, as well as the AGE pyrraline and pentosidine [15, 16]. However, urinary CML levels did not decrease significantly during this washout period [14]. The intake of Pretzel sticks containing high levels of CML and pyrraline increased urinary pyrraline excretion but did not significantly modulate urinary CML excretion. A recent study by Dittrich *et al.* [17] in newborn babies fed either low-CML breast milk or high-CML infant formulas showed that the urinary CML excretion was not influenced by the amount of CML ingested. From these studies, it appears that CML is not well absorbed in humans.

Additional support for limited CML bioavailability in humans come from a study of the trans-epithelial flux of Maillard reaction products in Caco-2 intestinal epithelial cell monolayers [18]. These studies showed that CML is not actively transported across the epithelial monolayer.

Together, these data suggest that, at least in humans, urinary CML excretion does not appear to be much influenced by dietary CML levels. It remains currently unknown whether this is due to CML not being well absorbed, it being metabolized, or whether it is "masked" by high endogenous CML levels.

Although CML is chemically quite inert it has been suggested that CML modified proteins may interact with RAGE (receptor for AGE) resulting in the activation of inflammatory responses [19].

Tests for cellular activation via RAGE generally employ glycated model proteins – in most cases albumin – generated by incubation with reducing sugars for several weeks at 37°C in a phosphate buffer. This results in multiple modifications of lysine and arginine residues, among which also CML. As a result, the effects observed by such a protein containing multiple modifications cannot necessarily be directly linked to the presence of CML in the protein since

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

other modifications could also contribute to the RAGE binding properties.

Therefore, the aim of the present study was to generate model proteins that are exclusively modified to CML in order to investigate their binding to RAGE as well as their ability to activate a cellular inflammatory response in cultured cells.

2 Materials and methods

2.1 Chemicals

Glyoxylic acid, lysine, β-lactoglobulin, HSA and $NaBH_3CN$ were purchased from Sigma-Aldrich (Buchs, Switzerland). The $^{13}C_6,^{15}N_2$-lysine (98% atom ^{13}C and 96–99% atom ^{15}N) was obtained from Cambridge Isotope Laboratories (Innerberg, Switzerland). CML and ^{13}C-labeled CML ($^{13}C_2$-CML) were prepared as reported elsewhere [20] using either glyoxylic acid or 1,2-$^{13}C_2$-glyoxylic acid (>99% atom ^{13}C) as starting material. All other reagents were from various commercial sources. S100B was prepared as described [21].

2.2 Generation of glycated proteins

The CML modified proteins were generated as described elsewhere [22]. Briefly, 500 mg of β-lactoglobulin (bLG) or HSA were dissolved in 150 mM phosphate buffer pH 7.4 containing 25 mM glyoxylic acid and 75 mM $NaBH_3CN$ in a total volume of 17.4 mL. After sterile filtration this mixtures were incubated at 50°C for 48 h. The reaction mixtures were diafiltrated (Labscale TFF System, Millipore, Zug, Switzerland) against 150 mM phosphate buffer pH 7.4 to remove unreacted glyoxylic acid and $NaBH_3CN$. Proteins incubated in 150 mM phosphate buffer pH 7.4 for 48 h at 50°C but without glyoxylic acid and $NaBH_3CN$ served as control. After dialysis, endotoxin was removed using Triton X-114 as described [23, 24]. The final preparations were devoid of endotoxin as tested with the limulus amoebocyte lysate (LAL) pyrogen test (Cambrex, BioConcept, Switzerland).

2.3 Generation of β-amyloid preparations

One milligram human β-amyloid (aa 1–40, Bachem, Bubendorf, Switzerland) was mixed in 1 mL PBS (Sigma-Aldrich, Buchs, Switzerland), sonicated and sterile filtered. The solution was incubated under stirring for 24 h at 37°C. A concentration of 10 μM was used for cell assays. The β-amyloid was tested negative for endotoxin.

2.4 Determination of lysine and CML content by LC-MS/MS

After a 4-h incubation in the presence of sodium borohydride protein samples (500 μg) were hydrolyzed with 6 N HCl (110°C, 24 h) as described by Drusch *et al.* [25]. Hydrolysates were supplemented with $^{13}C_2$-CML and $^{13}C_6,^{15}N_2$-lysine prior to purification by SPE with Oasis HLB 200 mg cartridges provided by Waters (Milford, MA, USA). Both lysine and CML were simultaneously determined by isotope dilution LC-MS/MS. The HPLC system consisted of an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) coupled to the TSQ Quantum mass spectrometer from ThermoFinnigan (San Jose, CA, USA). The column was an Atlantis dC18, 3 μm, 2.1 × 150 mm from Waters and the flow rate was set at 200 μL/min. The separation of lysine and CML was achieved with the following gradient using nonafluoropentanoic acid 5 mM in water as solvent A and nonafluoropentanoic acid 5 mM in ACN in solvent B: from 0 to 15 min: linear gradient from 85/15 (A/B, v/v) to 15/85 (A/B); from 15 to 20 min: 15/85 (A/B); from 21 min on: back to 85/15 (A/B). With such conditions, typical retention times were 9.9 min for lysine and 7.1 min for CML. The detection was carried out by positive ESI and the source parameters were selected as follows: spray voltage: 4.5 kV; capillary temperature: 300°C; collision pressure: 1.2 mTorr. The data acquisition was performed with a scan time at 40 ms. The chromatographic profile was recorded in the selected reaction monitoring mode, and two characteristic transitions were monitored per compound in order to improve the selectivity. Transitions were: lysine: quantifier: m/z 147 → 84 (collision energy at 15 eV); qualifier: m/z 147 → 130 (collision energy at 13 eV); $^{13}C_6,^{15}N_2$-lysine: quantifier: m/z 155 → 90; qualifier: m/z 155 → 137; CML: quantifier: m/z 205 → 84 (collision energy at 22 eV); qualifier: m/z 205 → 130 (collision energy at 13 eV); $^{13}C_2$-CML: quantifier: m/z 207 → 84; qualifier: m/z 207 → 130.

2.5 Purification of GST-sRAGE

The vector pHGST.2T vector containing the extracellular domain of human RAGE, sRAGE, was kindly provided by Sylvian Meloche (University of Montreal, Canada). Transformed *Escherichia coli* BL21(De3) cells were grown to OD = 1 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Eurogentec, Geneva, Switzerland). After overnight incubation at 30°C, the bacterial cells were harvested and resuspended in PBS containing protease inhibitors (complete protease inhibitor cocktail, Roche Diagnostics, Basel, Switzerland). The bacterial cells were sonicated and the cytoplasmic extract was collected by centrifugation for 30 min at 20 000 × *g*. The cytoplasmic extract was loaded onto a glutathione Sepharose 4B column (Amersham Biosciences Europe, Otelfingen, Switzerland) previously equilibrated with PBS. After washing with 10 column volumes, the GST-sRAGE was eluted with 10 mM glutathione (Sigma-Aldrich, Buchs, Switzerland). The protein concentration of the eluted protein was determined using the Bradford assay (Bio-Rad, Reinach, Switzerland).

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Genes on low-density arrays

Gene symbol	Gene name	Assay ID
18S	18S ribosomal RNA	4342379-18S
ActB	β-Actin	Hs99999903_m1
AGTR-1	Angiotensin II receptor, type 1	Hs00241341_m1
AKR1B1	Aldo-keto reductase family 1, member B1	Hs00739326_m1
CD62L/SELE	E selectin	Hs00174057_m1
COX-2/PTGS2	Cyclo-oxygenase 2	Hs00153133_m1
CXCL1/GROa	Chemokine (C-X-C motif) ligand 1	Hs00236937_m1
eNOS	Nitric oxide synthase 3 (endothelial)	Hs00167166_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
γGCS	γ-Glutamylcysteine ligase	Hs00155249_m1
GST-P1	Glutathione S-transferase P1	Hs00168310_m1
HO-1	Heme oxygenase 1	Hs00157965_m1
Hsp70	70kDa Heat shock protein 4	Hs00382884_m1
IL6	Interleukin 6	Hs00174131_m1
IL8	Interleukin 8	Hs00174103_m1
MCP-1/CCL2	Macrophage chemoattractant protein 1	Hs00234140_m1
RAGE	Receptor for AGE	Hs00153957_m1
SOCS-1	Suppressor of cytokine signaling 1	Hs00705164_s1
SOCS-3	Suppressor of cytokine signaling 3	Hs00269575_s1
SOD2	Mn-superoxide dismutase (mitochondrial)	Hs00167309_m1
TGFß1	Transforming growth factor beta 1	Hs00171257_m1
TNFα	Tumor necrosis factor alpha	Hs00174128_m1
VCAM-1	Vascular cell adhesion molecule 1	Hs00365486_m1
VEGF	Vascular endothelial growth factor	Hs00173626_m1

2.6 Immobilization of GST antibody on the CM5 sensor chips and test of control and CML proteins

Antibody against GST (Biacore, Uppsala, Sweden) was immobilized to CM5 sensor chip according to the previously described procedures [26]. Following immobilization of the antibody, binding of recombinant GST (Biacore) was tested to the surface as positive control. Through all the experiments, the flow rate was set-up at 50 μL/min. GST-sRAGE (75 μg/mL) was first injected over the flow cell to be captured by the GST antibody, resulting in an increase of about 1000 RU. The bound GST-sRAGE was then stable for over 20 min because of the slow dissociation of GST from the antibody. LPS-free, recombinant human S100B was purified as described [21]. Between each injection, the surface was regenerated using glycine buffer (pH 2.2) to dissociate both the compound and GST-RAGE from the antibody. Recombinant human S100B (10 μM) diluted in Tris-buffered saline containing 5 mM $CaCl_2$ was used as positive control. The glycated and control proteins were injected over the flow cells at 10 μM each. The Biacore affinity measurements for S100B were performed using a set of five twofold dilutions (5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.312 μM).

2.7 Cell culture and treatments

The human lung epithelial cell line Beas2b (ATCC CRL-9609, LGC Promochem, Molsheim, France) was cultured in bronchial epithelial growth medium (BEGM, Cambrex – BioConcept, Allschwil, Switzerland) on pre-coated IWAKI plates (Milian, Plan-les-Ouates, Switzerland). When cells were 80–90% confluent they were incubated for 6 h with 10 μM CML modified β-lactoglobulin, non-glycated β-lactoglobulin in BEBM medium or medium without any additions as controls. Cells were then rinsed twice with ice-cold PBS scratched off the plates and collected in PBS. The collected cells were centrifuged 5 min at $500 \times g$ at 4°C and the dry pellets were frozen at −80°C for later RNA isolation.

2.8 Determination of RNA expression

For real-time PCR, RNA was isolated from a pool of two wells of a 6-well plate using the Trizol method (Invitrogen, Basel, Switzerland). One microgram of total RNA was reverse-transcribed into cDNA using random hexamers and Multiscribe RT according to the manufacturer's protocol (Applied Biosystems, Rotkreuz, Switzerland). For each sample, 100 ng of cDNA were used for real-time PCR employing Applied Biosystems Low-Density Arrays (LDA) pre-configured with the primers and probe sets listed in Table 1 or using pre-made primer-probe sets for individual genes. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and analyzed with the SDS software package version 2.1 (Applied Biosystems). The mRNA expression for each target gene was normalized to the mRNA expression of glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

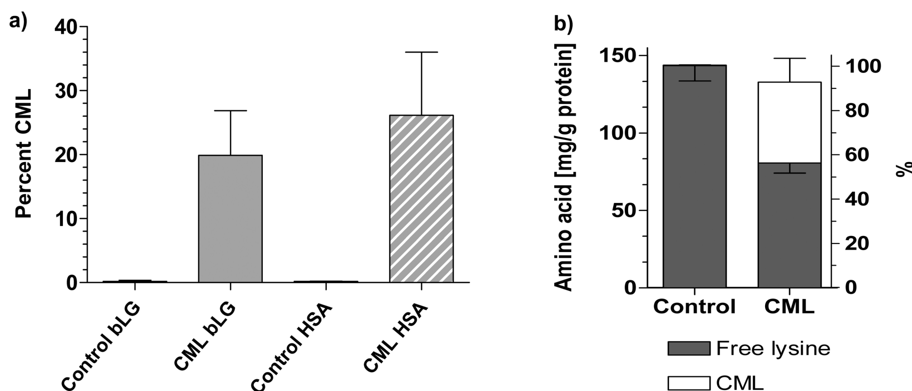

Figure 2. (A) Compositional analysis of CML-modified bLG and HSA. The composition of lysine and CML was determined by LC-MS/MS after acid hydrolysis. In total, three different CML-bLG and two different CML-HSA preparations were generated and tested together with four different control bLG and two different control HSA preparations. The data are shown as the means and SEM of independent glycation reactions done under identical conditions. (B) The balance of total lysine and CML in four control and two CML-modified bLG preparations. The left axis shows the amount of each amino acid in mg/g protein, the left axis the% modification. Error bars show the SEM, downward for lysine, upward fro CML.

2.9 Statistical analysis

Data were analyzed using the GraphPad Prism software (v 4.03, GraphPad Software, San Diego, USA). Statistical analyses included one-way ANOVA followed by Bonferroni post-tests or *t*-tests to compare two sets of treatment. Values are considered significant at $p < 0.05$.

3 Results

Glycation of bLG or HSA with glyoxylic acid in the presence of $NaBH_3CN$ resulted in a lysine to CML modification of between 15 and 40% (Fig. 2) as determined by isotope dilution LC-MS/MS. HSA was slightly more modified probably because of the relative greater number of lysine residues in this molecule compared to bLG. Figure 2B shows the average composition of the bLG model proteins. The loss in lysine residues in the modified proteins can be accounted for by the appearance of CML. Thus, under these conditions lysine is modified exclusively to CML as no other lysine modifications were detected (*e.g.* fructosyllysine and pyrraline, data not shown).

Unlike preparations incubated in the presence of lactose or glucose (not shown) these preparation were colorless and did not show significantly altered absorbance or fluorescence compared to control incubated bLG or HSA (Figs. 3A and B). SDS-PAGE analysis revealed that the CML modified bLG and HSA did not show a significant shift in molecular weight of the major bands at about 20 and 66 kDa, respectively (Fig. 4). The addition of 5 mol CML/mol bLG (30% lysine modification) will increase the molecular weight by 730, probably too little to be detected on SDS-PAGE. Together, these data show that the incubation of bLG or HSA with glyoxylic acid and $NaBH_3CN$ resulted in proteins in which CML was by far the predominant lysine modification.

Figure 5 shows representative binding curves for the RAGE binding of two CML-glycated bLG protein preparations compared to S100B and unglycated bLG using the Biacore technique. Injection of 10 μM S100B over the sensor chip resulted in the binding of the calcium binding protein to antibody-captured GST-RAGE as seen on the sensorgram (short dashed line, Fig. 5). For compounds that show binding at 10 μM (*e.g.* S100B), measurements were performed using dilutions from 0.3125 to 5.0 μM to determine the binding affinity. It should be noted that this was done in a buffer containing 5 mM $CaCl_2$ in order to enhance dimerization of the S100B proteins. No binding was detected when 10 μM unglycated bLG (solid back line, Fig. 5), or two independent preparations of CML-modified bLG (10 μM, long dashed lines, Fig. 5) were injected onto the surface. Because no binding was observed concentrations lower than 10 μM were not tested. The positive control S100B, an endogenous RAGE ligand, bound to RAGE with a dissociation constant of approximately 0.50 μM while the unglycated bLG or HSA as well as the CML-modified bLG or HSA did not bind to RAGE. In total, four different control bLG and three different bLG-CML preparations as well as two preparations each of control and CML HSA were tested and none showed any RAGE binding. These results demonstrate that a CML modified protein does not possess the correct tertiary structure or modification to make it a RAGE ligand under these experimental conditions.

Because CML-bLG or CML-HSA binding to RAGE using the Biacore system did not show any binding affinity for RAGE, it can be assumed that these CML-modified proteins would not show any cellular activation by RAGE in a cell culture system of cells expressing RAGE. The expres-

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

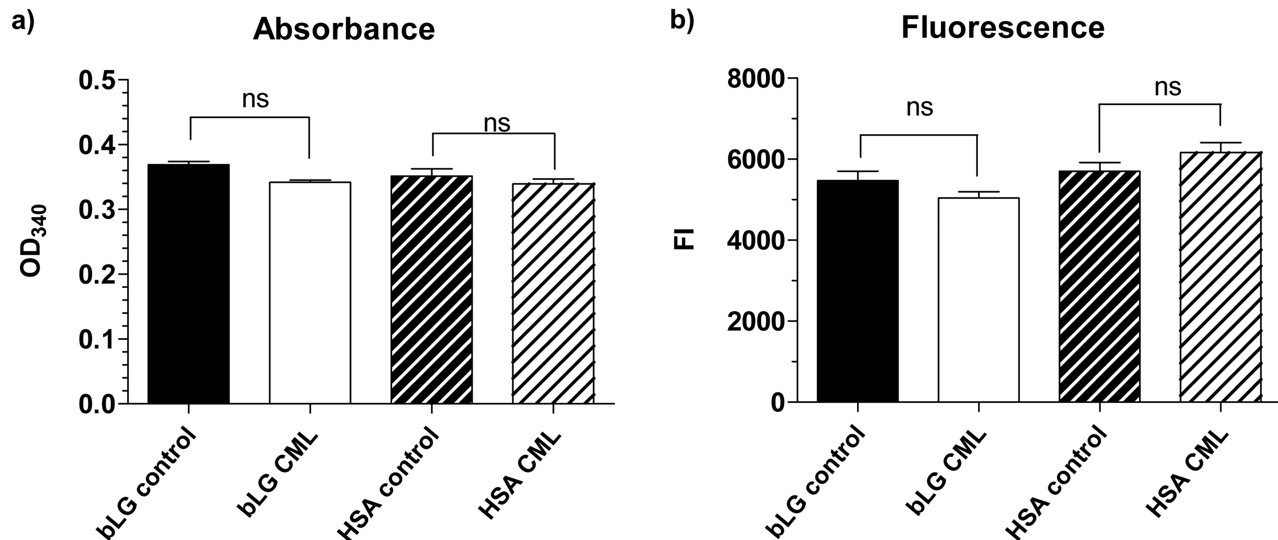

Figure 3. Absorbance and fluorescence of CML-modified bLG and HSA. Absorbance (A) was determined at 340 nm, fluorescence (B) at λ_{ex} = 340/λ_{em} = 430. The data shown are the means and SD of several independent glycation reactions done under identical conditions (ten CML control, four bLG-CML, six HSA control and two HSA-CML). *ns* Indicates that no statistically significant differences between the control and CML preparations were observed. Values represent means and SD.

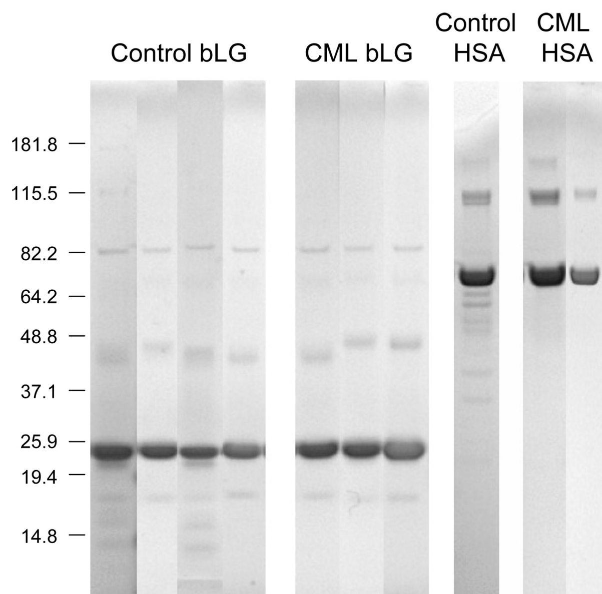

Figure 4. SDS-PAGE analysis of different bLG and HSA control and glycation reactions. One control HSA preparation is not shown.

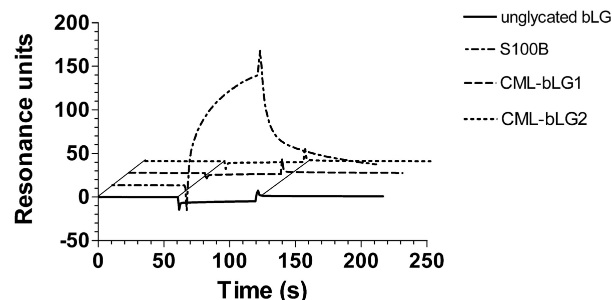

Figure 5. Binding curves of S100B and glycated bLG proteins to RAGE using the Biacore technique. Injection of 10 μM S100B over the sensor chip resulted in the binding of the calcium binding protein to antibody-captured GST-RAGE as seen on the sensorgram (point-dash-point line). No binding was detected when 10 μM unglycated bLG (solid black line), or two different preparations of CML-modified bLG (long and short dashed lines) were injected. This figure is representative of at least three independent experiments.

sion of pro-inflammatory marker mRNA was analyzed by Taq-Man real-time quantitative PCR (Fig. 6) in the lung epithelial cell line BEAS-2b that was verified to express RAGE (data not shown). A low-density array containing the 21 genes listed in Table 1 as well as individual primer/probe sets for IL6, IL8 and TNFα was used. Figure 6 shows that both TNFα and the RAGE ligand β-amyloid [27] consistently induced the expression of IL6, IL8 and TNFα. Of note is the observation that the induction of gene expression by β-amyloid was much lower than by TNFα. The expression analysis of the low-density arrays showed that, in addition to these three inflammatory markers, GROα, VCAM-1 and SOD2 were also consistently induced by TNFα and β-amyloid (not shown). The combined data show that the BEAS-2b cells reacted to pro-inflammatory simulation induced by either TNFα or the RAGE ligand β-amyloid.

As expected, unglycated bLG was not able to stimulate the expression of any of the genes analyzed (Fig. 6). Our results show that also the CML-modified bLG was unable to induce the expression of inflammatory genes. This is in agreement with the Biacore data and underlines the fact

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

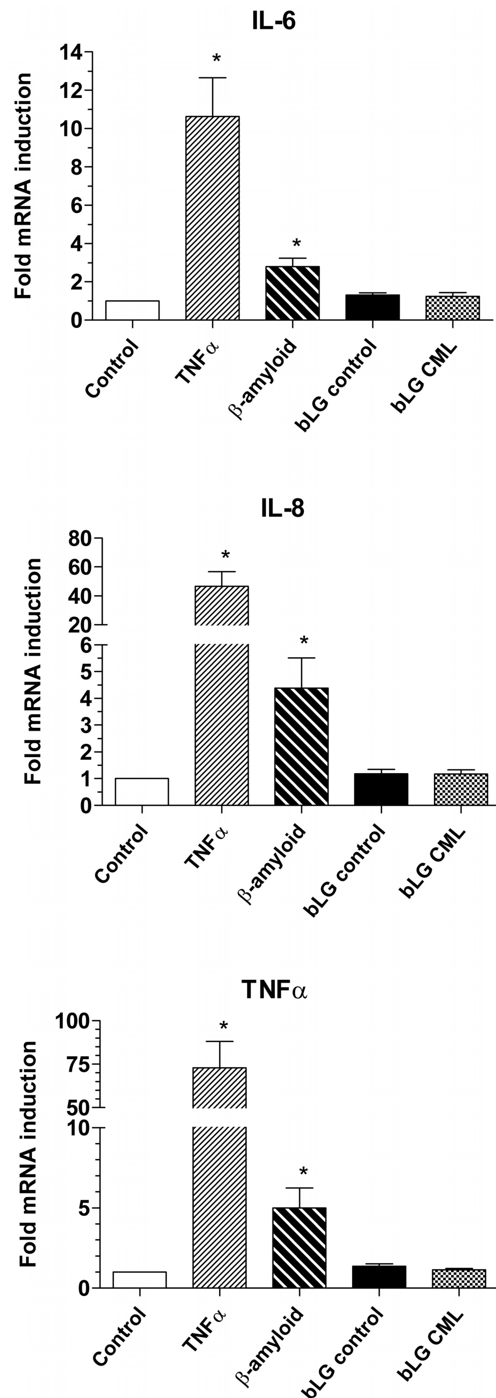

Figure 6. Induction of IL6, IL8 and TNFα mRNA expression in BEAS-2b lung epithelial cells. Cells were treated when confluent in medium containing 5% FBS for 6 h. TNFα (1 ng/mL) served as positive control for activation of inflammatory gene expression, β-amyloid preparations (10 μM) as positive control for RAGE activation. Messenger RNA expression was normalized to GAPDH expression. Cells were treated with 10 μM unmodified or modified bLG. The data are shown as the means and SEM of at least six independent analyses. The asterisks indicate that values are significantly different from control at $p < 0.05$.

that CML-bLG is unable to interact with RAGE to induce cell activation under our experimental conditions. These data further show that the CML-modified bLG is not able to activate other potential receptors involved in inflammation that may be expression in BEAS-2b cells.

Thus, in contrast to the consensus that CML is a RAGE ligand, our data demonstrate that CML-containing bLG or HSA are not able to bind to RAGE. CML-modified bLG also cannot activate a RAGE-signaling cascade. Preliminary tests in the human retinal epithelial cell line ARPE-19 also did not show any cellular activation by the CML-modified bLG preparations.

4 Discussion

In the present work, we demonstrate that two model proteins (bLG and HSA) that were chemically modified to contain between 15 and 40% CML (and no other lysine modification based on lysine and CML quantification) were unable to bind to RAGE. The use of glyoxylic acid in the presence of $NaCNBH_3$ for the glycation reaction of lysine residues in proteins results in an almost exclusive formation of CML as shown earlier [20].

CML-modified bLG was also unable to activate RAGE-mediated cell signaling pathways in the human lung epithelial cell line Beas2B. This is in contrast to several reports [19, 28] that claim CML to be a major RAGE ligand. However, our data are in agreement with data published by Valencia *et al.* [22, 29], Ballinger *et al.* [30], Lieuw-a-Fa *et al.* [31] and Wilton *et al.* [32]. Valencia *et al.* showed that CML-modified BSA was not able to compete for cellular binding of [^{125}I]ribose-BSA [29] while binding was competed for by ribose-, fructose- or glucose-BSA with decreasing efficiency. In addition, they demonstrated that even though some AGE-modified protein preparations were able to bind to cells, apparently via RAGE, this was not sufficient to stimulate inflammatory signaling as monitored by TNFα secretion or VCAM-1 expression on human microvascular endothelial cells (HMEC-4). The positive control for these experiments was the endogenous RAGE ligand S100B [33–35].

Ballinger *et al.* [30] showed that vascular smooth muscle cells could not be stimulated by CML-modified BSA or AGE-BSA at concentrations ranging from 1 to 100 μg/mL as determined by a stimulation of glucose consumption or ^{35}S-methionine/cysteine incorporation into newly synthesized proteins. Because these authors concluded that RAGE expression on these cells was probably too low to elicit any response these data cannot be taken as proof that CML does not bind to RAGE.

Lieuw-a-Fa *et al.* [31] showed that neither [^{125}I]CML-, nor [^{125}I]methylglyoxal-modified HSA were able to bind to endothelial cells. Lastly, also using the Biacore technique Wilton *et al.* [32] showed that RAGE expressed in *E. coli*

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

was unable to bind to native or CML-glycated BSA immobilized on the sensor chip while it did bind to immobilized AGE-modified BSA generated by incubation with ribose. Together, these data support our finding that CML-modified proteins are unable to bind to RAGE.

How can the discrepancy between the results presented here and cited above be reconciled with the mass of literature claiming CML to be a RAGE ligand? The simplest explanation is that for most studies investigating AGE-RAGE interactions heated protein-sugar mixes were employed that resulted in multiple modifications. These were in general not characterized apart from isolated analyses for CML content. Under the experimental conditions used (incubation in phosphate buffer at 37°C for several weeks) many protein modifications/glycations take place, one of which is the formation of CML. While these preparations were able to bind to RAGE, this may not have been due to the presence of CML on the protein but rather to other structures generated during protein glycation that facilitated RAGE binding. This contention is supported by our unpublished observations as well as the work of Valencia *et al*. [29] and Wilton *et al.* [32] showing that ribose, glucose or glycolaldehyde modified proteins were able to bind to RAGE but not CML-modified proteins.

As discussed in Wilton *et al.* [32], it could be argued that CML-modified proteins do not bind to RAGE because RAGE was expressed in bacteria and may lack the necessary post-translational modifications/glycosylations necessary for this interaction. However, this is unlikely because studies with the RAGE ligand S100B have shown that it binds to RAGE independently of the glycosylation state of RAGE [36]. Furthermore, the data of Valencia *et al.* [29] show that other AGE modifications such as induced by ribose or glycolaldehyde do bind to RAGE. Our preliminary data also show that other protein modifications, such as those induced by glycolaldehyde or other dicarbonyls, will form structures capable of RAGE binding (unpublished observations). The fact that we and Valencia *et al.* [29] did not find any cellular activation by CML-modified proteins can be interpreted that such modified proteins are unable to bind to RAGE in its glycosylated form present on mammalian cells. They further suggest that CML-modified proteins are also unable to stimulate other potential signaling pathways that could lead to inflammatory protein expression.

In summary, our data show that proteins modified to primarily contain CML (modification of up to 40% of lysine residues) are not RAGE ligands. The results of earlier studies claiming CML to be RAGE ligands [19] may be explained by the fact that protein modifications generated with reducing sugars after an extended incubation at 37°C will result in other modifications that may be the true ligands for RAGE. Because CML is not a selective AGE marker and appears to be unable to bind to and activate RAGE, the currently available data based on CML analyses may need to be carefully reassessed in light of these facts.

The authors have declared no conflict of interest.

5 References

[1] Henle, T., Miyata, T., Advanced glycation end products in uremia. *Adv. Ren. Replace. Ther.* 2003, *10*, 321–331.

[2] Thorpe, S. R., Baynes, J. W., Maillard reaction products in tissue proteins: new products and new perspectives. *Amino Acids* 2003, *25*, 275–281.

[3] Brownlee, M., Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001, *414*, 813–820.

[4] Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., *et al.*, The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* 1996, *271*, 9982–9986.

[5] Saxena, P., Saxena, A. K., Cui, X. L., Obrenovich, M., *et al.*, Transition metal-catalyzed oxidation of ascorbate in human cataract extracts: possible role of advanced glycation end products. *Invest. Ophthalmol. Vis. Sci.* 2000, *41*, 1473–1481.

[6] Januszewski, A. S., Alderson, N. L., Metz, T. O., Thorpe, S. R., Baynes, J. W., Role of lipids in chemical modification of proteins and development of complications in diabetes. *Biochem. Soc. Trans.* 2003, *31*, 1413–1416.

[7] Kamalvand, G., Ali-Khan, Z., Immunolocalization of lipid peroxidation/advanced glycation end products in amyloid A amyloidosis. *Free Radic. Biol. Med.* 2004, *36*, 657–664.

[8] Muntané, G., Dalfó, E., Martínez, A., Rey, M. J., *et al.*, Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease. *J. Neurochem.* 2006, *99*, 177–185.

[9] Figarola, J. L., Scott, S., Loera, S., Xi, B., *et al.*, Prevention of early renal disease, dyslipidaemia and lipid peroxidation in STZ-diabetic rats by LR-9 and LR-74, novel AGE inhibitors. *Diabetes Metab. Res. Rev.* 2005, *21*, 533–544.

[10] Goldberg, T., Cai, W., Peppa, M., Dardaine, V., *et al.*, Advanced glycation end products in commonly consumed foods. *J. Am. Diet. Assoc.* 2004, *104*, 1287–1291.

[11] Bergmann, R., Helling, R., Heichert, C., Scheunemann, M., *et al.*, Radio fluorination and positron emission tomography (PET) as a new approach to study the in vivo distribution and elimination of the advanced glycation endproducts N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL). *Nahrung* 2001, *45*, 182–188.

[12] Ahmed, N., Mirshekar-Syahkal, B., Kennish, L., Karachalias, N., *et al.*, Assay of advanced glycation endproducts in selected beverages and food by liquid chromatography with tandem mass spectrometric detection. *Mol. Nutr. Food Res.* 2005, *49*, 691–699.

[13] Somoza, V., Wenzel, E., Weiss, C., Clawin-Radecker, I., *et al.*, Dose-dependent utilisation of casein-linked lysinoalanine, N(epsilon)-fructoselysine and N(epsilon)-carboxymethyllysine in rats. *Mol. Nutr. Food Res.* 2006, *50*, 833–841.

[14] Foerster, A., *Mathematik und Naturwissenschaften*, Technische Universität Dresden, Dresden 2006, pp. 181.

[15] Foerster, A., Henle, T., Glycation in food and metabolic transit of dietary AGEs (advanced glycation end-products): studies on the urinary excretion of pyrraline. *Biochem. Soc. Trans.* 2003, *31*, 1383–1385.

[16] Foerster, A., Kühne, Y., Henle, T., Studies on absorption and elimination of dietary maillard reaction products. *Ann. N. Y. Acad. Sci.* 2005, *1043*, 474–481.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[17] Dittrich, R., Hoffmann, I., Stahl, P., Muller, A., *et al.*, Concentrations of Nepsilon-carboxymethyllysine in human breast milk, infant formulas, and urine of infants. *J. Agric. Food Chem.* 2006, *54*, 6924–6928.

[18] Grunwald, S., Krause, R., Bruch, M., Henle, T., Brandsch, M., Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems. *Br. J. Nutr.* 2006, *95*, 1221–1228.

[19] Kislinger, T., Fu, C., Huber, B., Qu, W., *et al.*, 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.* 1999, *274*, 31740–31749.

[20] Delatour, T., Fenaille, F., Parisod, V., Vera, F. A., Buetler, T., Synthesis, tandem MS- and NMR-based characterization, and quantification of the carbon 13-labeled advanced glycation endproduct, 6-N-carboxymethyllysine. *Amino Acids* 2006, *30*, 25–34.

[21] Ostendorp, T., Heizmann, C. W., Kroneck, P. M., Fritz, G., Purification, crystallization and preliminary X-ray diffraction studies on human Ca^{2+}-binding protein S100B. *Acta Crystallograph. F* 2005, *61*, 673–675.

[22] Valencia, J. V., Weldon, S. C., Quinn, D., Kiers, G. H., *et al.*, Advanced glycation end product ligands for the receptor for advanced glycation end products: biochemical characterization and formation kinetics. *Anal. Biochem.* 2004, *324*, 68–78.

[23] Bordier, C., Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 1981, *256*, 1604–1607.

[24] Liu, S., Tobias, R., McClure, S., Styba, G., *et al.*, Removal of endotoxin from recombinant protein preparations. *Clin. Biochem.* 1997, *30*, 455–463.

[25] Drusch, S., Faist, V., Erbersdobler, H. F., Determination of N^{ε}-carboxymethyllysine in milk products by a modified reversed-phase HPLC method. *Food Chem.* 1999, *65*, 547–553.

[26] Johnsson, B., Lofas, S., Lindquist, G., Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 1991, *198*, 268–277.

[27] Du Yan, S., Zhu, H., Fu, J., Yan, S. F., *et al.*, Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 1997, *94*, 5296–5301.

[28] Hudson, B. I., Bucciarelli, L. G., Wendt, T., Sakaguchi, T., *et al.*, Blockade of receptor for advanced glycation endproducts: a new target for therapeutic intervention in diabetic complications and inflammatory disorders. *Arch. Biochem. Biophys.* 2003, *419*, 80–88.

[29] Valencia, J. V., Mone, M., Koehne, C., Rediske, J., Hughes, T. E., 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* 2004, *47*, 844–852.

[30] Ballinger, M. L., Thomas, M. C., Nigro, J., Ivey, M. E., *et al.*, Glycated and carboxy-methylated proteins do not directly activate human vascular smooth muscle cell. *Kidney Int.* 2005, *68*, 2756–2765.

[31] Lieuw-a-Fa, M. L., Schalkwijk, C. G., Engelse, M., van Hinsbergh, V. W., 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.* 2006, *95*, 320–328.

[32] Wilton, R., Yousef, M. A., Saxena, P., Szpunar, M., Stevens, F. J., Expression and purification of recombinant human receptor for advanced glycation endproducts in Escherichia coli. *Protein Expr. Purif.* 2006, *47*, 25–35.

[33] Donato, R., S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int. J. Biochem. Cell Biol.* 2001, *33*, 637–668.

[34] Hofmann, M. A., Drury, S., Fu, C., Qu, W., *et al.*, RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 1999, *97*, 889–901.

[35] Ramasamy, R., Vannucci, S. J., Shi Du Yan, S., Herold, K., *et al.*, Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* 2005, *15*, 16R–28R.

[36] Ostendorp, T., Weibel, M., Leclerc, E., Kleinert, P., *et al.*, Expression and purification of the soluble isoform of human receptor for advanced glycation end products (sRAGE) from Pichia pastoris. *Biochem. Biophys. Res. Commun.* 2006, *347*, 4–11.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim