RESEARCH ARTICLE

Glycolaldehyde-modified β-lactoglobulin AGEs are unable to stimulate inflammatory signaling pathways in RAGE-expressing human cell lines

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Scope: Advanced glycation endproducts (AGEs) are suspected to stimulate inflammatory signaling pathways in target tissues *via* activation of the receptor for AGEs. Endotoxins are generally recognized as potential contamination of AGE preparations and stimulate biological actions that are very similar as or identical to those induced by AGEs.

Methods and results: In our study, we used glycolaldehyde-modified β-lactoglobulin preparations as model AGEs and employed two methods to remove endotoxin using either affinity columns or extraction with Triton X-114 (TX-114). Affinity column-purified AGEs retained their ability to stimulate inflammatory signaling as measured by mRNA expression of inflammatory cytokines in the human lung epithelial cell line Beas2b. However, glycolaldehyde-modified AGEs purified by extraction with TX-114 did not show any stimulation of mRNA expression of inflammatory cytokines. The presence of a cell stimulating endotoxin-like activity was demonstrated in the detergent phase after extraction with TX-114, thus indicating that not AGEs but a lipophilic contamination was responsible for the stimulation of inflammatory signaling.

Conclusion: Our results demonstrate that glycolaldehyde-modified AGEs are unable to induce inflammatory signaling in receptor for AGE-expressing cells. The observed cell-activating activity can be ascribed to an endotoxin-like lipophilic contamination present in AGE preparations and affinity column purification was insufficient to remove this contamination.

Keywords:
Advanced glycation endproducts / Endotoxin / Inflammatory cytokines / Receptor for AGE / Triton X-114

1 Introduction

Advanced glycation endproducts (AGEs) are perceived as having adverse health effects. Mechanistically, this argument is based on the observations that AGEs can bind to and appear to activate the receptor for AGEs – receptor for AGE (RAGE) – that is linked to inflammation and disease [1–6]. RAGE is a receptor of the immunoglobulin family with a variable and two constant regions linking the extracellular domain to a short, charged intracellular domain *via* a single trans-membrane domain. The highest RAGE expression in adult tissues has been found in lung, skeletal muscle, heart and liver [7]. Endothelial cells, monocytes, mesangial and smooth muscle cells were also shown to express RAGE [7]. Ligand binding to RAGE results in free radical production, activation of MAP kinase pathways and stimulation of cytokine production and, in endothelial cells, vascular adhesion molecules [3, 5]. There are several endogenous RAGE ligands including the S100 calcium-binding

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Abbreviations: AGEs, advanced glycation endproducts; **bLG,** beta-lactoglobulin; **GA,** glycolaldehyde; **GST,** glutathione S-transferase; **RAGE,** receptor for AGEs; **TX-114,** Triton X-114

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proteins [8, 9], amphoterin or high-mobility group protein 1 (HMGB-1 [10]) and β-amyloid [1, 11].

Several publications have plausibly shown that AGEs prepared by incubating proteins with reducing sugars such as glucose, fructose or ribose form novel structures that bind to RAGE and seem able to stimulate the expression of inflammatory mediators. These protein-bound AGEs are a heterogeneous group of chemically distinct modifications that include simple lysine modifications such as carboxymethyl- or carboxyethyllysine, but also complex intra- and intermolecular cross-links [12–14]. Recent studies have shown that AGEs interact with the V-domain of RAGE [15, 16]. The study by Xie *et al.* [16] has shown that the early glycation Amadori product fructoselysine does not bind to RAGE. On the other hand, Xie *et al.* [16] have shown that complex AGEs with intramolecular cross-links bind to RAGE with high affinity probably by recruiting interaction surfaces on different receptor subunits in grouped receptor oligomers [16]. This is consistent with the data of Ostendorp *et al.* [17] who showed that oligomeric forms of S100B bind to RAGE with higher affinity than dimeric forms.

Despite the overwhelming literature showing that the AGE-RAGE interaction results in cell activation, there are a few reports that question this view. Using competition of various AGEs with ^{125}I-labeled ribose-BSA to cell-expressed RAGE, Valencia *et al.* [18, 19] demonstrated that various AGEs bound specifically to endothelial cells. Although CML-modified BSA was unable to compete for radiolabeled ribose-BSA, glucose-, fructose-, ribose- and glycolaldehyde (GA)-modified BSA were able to displace the radiolabeled ribose BSA, supporting the proposition that cross-linked AGEs are good RAGE ligands. However, the data of Valencia *et al.* [18, 19] further showed that RAGE-binding of these different AGEs was not sufficient to stimulate inflammatory signaling, whereas the endogenous RAGE ligand S100B stimulated both VCAM-1 and TNF-α expression in HMEC-4 cells [18].

Similar findings were reported by Reznikov *et al.* [20] showing that strictly endotoxin-free AGEs were unable to stimulate cytokine production (TNF-α, IL-1β and IL-8) in peripheral blood monocytes of healthy donors. However, low endotoxin concentrations in the AGE preparations significantly augmented the synthesis and release of pro-inflammatory cytokines from peripheral blood monocytes. This study highlights the importance of endotoxin contamination as primary inflammatory stimulus present in AGE preparations. Most studies published over the past few years have, thus, employed endotoxin removal resins such as DetoxiGel or EndoTrap to purify AGE preparations.

Extraction with Triton X-114 (TX-114) has recently been described as an alternative method for the removal of endotoxin from biological preparations [21–24]. This method is based on the property of TX-114 to form micelles when warmed above 30°C [25]. After phase separation, hydrophilic molecules separate into the aqueous phase, whereas lipophilic molecules separate into the detergent phase. Thus, lipophilic endotoxin can effectively be separated from hydrophilic proteins in solution [23]. One possible advantage of TX-114 over other methods of endotoxin removal may be that the detergent aids in the dissociation of endotoxin from the target protein [26].

Despite the evidence that at least some AGEs bind to RAGE with high affinity, it is not clear whether RAGE engagement will consistently result in inflammatory cell activation. Here, we investigated the cell activation of AGE preparations generated by chemical modification of β-lactoglobulin (bLG) with GA that results in protein cross-links. We used affinity column purification and TX-114 extraction to remove endotoxin. The purified preparations were then tested for their capacity to activate inflammatory signaling in the human lung epithelial cell line Beas2b and the human retinal pigment epithelial cell line ARPE19.

2 Materials and methods

2.1 Chemicals

GA, isopropyl-β-D-thiogalactopyranosid and TX-114 were purchased from Sigma-Aldrich-Fluka (Buchs, Switzerland), bLG was from either Davisco (Le Sueur, MN, USA) or Sigma-Aldrich, S100B was from Calbiochem (VWR International, Dietikon, Switzerland), the DetoxiGel columns were from Pierce (Perbio Science Switzerland SA, Lausanne, Switzerland), the P20 polyoxyethylenesorbitan detergent was from Biacore AB (Uppsala, Sweden) and the PyrogenT gel clot assay was from Lonza (BioConcept, Allschwil, Switzerland). The anti-RAGE antibody used for Western blots was generated as described previously [27].

2.2 Generation of GA-modified bLG

To produce GA-modified bLG, GA was added to a solution of 30 mg/mL bLG in 50 mM HEPES buffer, pH 7.4, to a final concentration of 1.7, 16.7 or 100 mM (1-, 10- and 60-fold molar excess of GA relative to bLG). We used high-quality HEPES (Sigma H4034, $<$ 5 ppm Fe and heavy metals) to reduce the possible contamination of transition metals in the incubation mix [20]. The solution was sterile filtered and incubated at 50°C for 48 h. The solution was extensively diafiltered (Pellicon Labscale, Millipore, Switzerland) against water to remove residual GA and the protein was recovered by lyophilisation after determination of the protein concentration spectrophotometrically using a NanoDrop ND1000 (Witec, Littau, Switzerland). The extent of glycation (lysine loss) as well as the composition of the modified amino acids CML, pyrraline and fructoselysine was determined as described previously [28].

2.3 Biacore

The extracellular part of RAGE fused to glutathione S-transferase (GST) was expressed and purified as described previously [17]. Briefly, the GST-RAGE fusion was cloned into the pHGST.2T plasmid and transformed into BL21(DE3) cells. Transformed BL21(DE3) cells were grown at 37°C in LB/ampicillin medium to an optical density of 1 at 600 nm. Cultures were then transferred to room temperature and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After overnight incubation, the cells were pelleted, resuspended in PBS containing protease inhibitors (Complete, Roche, Mannheim, Germany) and lyzed by sonication. The clarified soluble fraction was loaded onto a glutathione sepharose column (Amersham Bioscience, Uppsala, Sweden), washed with PBS, and GST-RAGE was eluted with 10 mM glutathione in 50 mM Tris, pH 8.0. The protein was dialyzed against PBS and concentrated. A molar extinction coefficient of 80 540 $M^{-1} cm^{-1}$ was used to calculate the protein concentration.

All surface plasmon resonance experiments were performed on a Biacore 3000 instrument (GE Healthcare, Piscataway, NJ, USA). GST-RAGE was captured with a mouse anti-GST antibody (GE Healthcare) covalently coupled to CM5 sensor chips as described previously [17]. A series of increasing S100B concentrations were injected over the flow-cells in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.005% P20. For each compound, a series of increasing concentrations ranging from 2.5 to 10 μM were applied to the flow cell. After each association/dissociation cycle, the surface was regenerated for 1 min with 10 mM HCl (pH 2). The sensorgrams were analyzed by global analysis using the BiaEvaluation 3.1 software [29]. About 1500 resonance units were obtained for each immobilization of GST-RAGE to anti-GST-treated CM5 surface.

2.4 Cell culture and treatments

The human lung epithelial cell line Beas2b was purchased from ATCC (LGC Promochem, Molsheim, France). Cells were cultured in bronchial epithelial growth medium (BEGM, Cambrex – BioConcept) on pre-coated IWAKI plates (Milian, Plan-les-Ouates, Switzerland). At 80% confluence, the cells were incubated for 1 h with 10 μM GA-modified bLG or non-glycated control bLG in BEGM or medium without any additions; 1 ng/mL TNF-α or 5 μM S100B served as positive controls. Cells were then rinsed with ice-cold Dulbecco's PBS (Sigma-Aldrich), scraped off the plates and collected in Trizol Reagent (Invitrogen, Basel, Switzerland). The collected cells were frozen at −80°C until RNA isolation.

ARPE-19 cells, a human retinal pigment epithelial cell line, were purchased from ATCC (LGC Promochem). The cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium (ATCC), supplemented with 10% fetal bovine serum. Cells were grown to 80% confluence and then incubated with bLG preparations like Beas2b cells.

All cells were incubated at 37°C in an atmosphere of 5% CO_2 in air.

2.5 Determination of RNA expression

For real-time PCR, RNA was isolated from a 6-well plate using the Trizol method according to the manufacturer's protocol (Invitrogen). One microgram of total RNA was reverse-transcribed to cDNA using random hexamers and Multiscribe RT according to the manufacturer's protocol (Applied Biosystems, Rotkreuz, Switzerland). For each sample, 50 ng of cDNA were used for real-time quantitative PCR, employing Applied Biosystems primer and probe sets for human TNF-α, IL-6 and IL-8 and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The raw data were analyzed with the SDS software version 1.1 (Applied Biosystems). The mRNA expression for each target gene was normalized to the mRNA expression of the human TATA-box binding protein (TBP, Hs00427620_m1).

2.6 Western blots

Untreated cells were cultured in 60-mm dishes at 37°C in an atmosphere of 5% CO_2. Cells were washed twice with PBS and collected in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Rotkreuz, Switzerland). The homogenates were collected by centrifugation at $8000 \times g$ for 10 min at 4°C and the protein concentration of the supernatant was determined with a Bio-Rad Protein assay kit (Bio-Rad, Reinach, Switzerland). Fifty micrograms of protein were loaded onto 4–12% NuPAGE-NOVEX Bis-Tris Pre-Cast Gels (Invitrogen). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Invitrogen) and probed with anti-RAGE antibody A11 (1:100, [27]). As secondary antibody, a sheep horseradish peroxidase-linked anti-mouse IgG (1:2000; GE Healthcare, Otelfingen, Switzerland) was used. ECL chemiluminescence system (Pierce) was used for visualization of the immunoblots.

2.7 Endotoxin removal and TX-114 extraction

Endotoxin was removed by repeated passage over polymyxin B-agarose columns (Detoxi-Gel AffinityPak prepacked columns; Pierce) according to the manufacturer's protocol.

In parallel experiments, extraction with TX-114 was used. TX-114 was added to the protein preparation to a final concentration of 1%. The mixture was incubated at 4°C for 10 min with constant stirring. The sample was then transferred to a 37°C water bath, incubated for 10 min and centrifuged (16 000 × *g*, 5 min) at 37°C. The upper aqueous phase containing the protein was carefully removed and subjected to TX-114 phase separation for at least two more cycles. Residual TX-114 was removed by three additional heating-centrifugation cycles without the addition of TX-114.

Residual endotoxin was determined using the Limulus Amebocyte Lysate PyrogenT Plus assay (BioConcept) according to the manufacturer's (Cambrex/Lonza) protocol.

2.8 Statistical analysis

Data are expressed as mean and standard deviation of biological triplicates. The data were analyzed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA) using ANOVA analysis of variation with Bonferroni post-tests or two-tailed Student's *t*-test. Data were considered significant at $p < 0.05$.

3 Results

We have shown previously that CML-modified bLG or HSA are unable to stimulate inflammatory signaling in the human lung epithelial cell line Beas2b [30]. Here, we first evaluated the RAGE expression in different human cell lines. Western blot analysis shows that Beas2b cells have the highest RAGE expression of the four cell lines investigated (Fig. 1), thus confirming our choice of this cell line to investigate RAGE-mediated cell activation. Other RAGE-expressing cell lines (ARPE-19, A431, HT-29) show detectable but much lower RAGE expression levels. Due to the slightly longer primary amino acid sequence of bovine compared with human RAGE (416 *versus* 404 aa) the bovine RAGE migrated slightly slower on SDS-PAGE (Fig. 1).

We then established the time course of mRNA induction of the inflammatory mediators TNF-α, IL-6 and IL-8 in Beas2b cells upon stimulation with both TNF-α (Fig. 2) and S100B (data not shown). The data show that TNF-α treatment of Beas2b cells resulted in a rapid stimulation of mRNA expression of the three cytokine genes tested that peaked after 1 h. Thereafter, the relative mRNA expression levels decreased but remained elevated above control levels for at least 6 h. A similar time-course was obtained after stimulation with S100B (data not shown). Therefore, all further experiments were performed with mRNA collected after 1 h of stimulation with TNF-α, S100B or AGEs. In all experiments, all three cytokine genes were analyzed (TNF-α, IL-6 and IL-8) but for simplicity results are shown only for

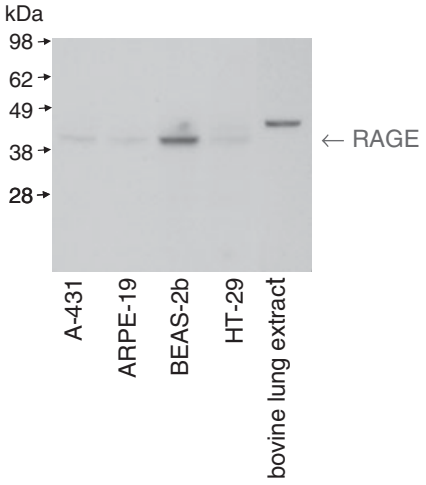


Figure 1. Western blot analysis of RAGE expression in different human cell lines. Fifty micrograms of total cell extract was loaded. Bovine lung extract served as positive control. Unglycosylated bovine RAGE has a MW of 44.2 kDa, human RAGE of 42.8 kDa (ExPASy.org).

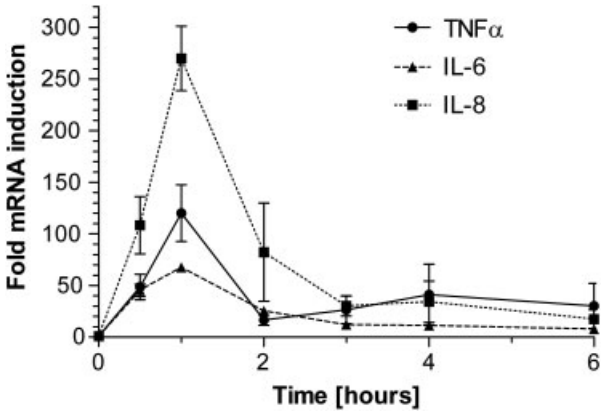
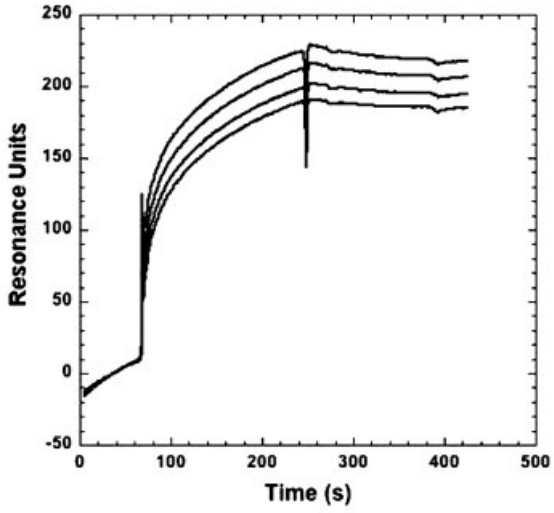


Figure 2. Time course of cytokine mRNA induction in Beas2b cells stimulated with TNF-α. Cells were incubated with 1 ng/mL TNF-α and mRNA expression was determined by real-time quantitative PCR using primer/probe sets for TNF-α, IL-6 and IL-8 relative to TATA box-binding protein as described in Section 2.

the induction of TNF-α expression. The mRNA expression/induction of the other two cytokines always followed the same pattern as observed for TNF-α.

Biacore-binding assays were performed to investigate the interaction of GA-modified bLG (bLG-GA) with bacterially expressed RAGE. Figure 3 shows association–dissociation curves for various bLG-GA concentrations. The association of bLG-GA with RAGE is very rapid followed by a slow dissociation. A kD of 139 nM was calculated from these data for the affinity of bLG-GA for RAGE.

Next, we tested whether different batches of bLG-GA AGE preparations would stimulate the expression of inflammatory mediators in Beas2b cells. Figure 4A shows



Protein	Glycation	Modification	RAGE binding K_D
βLG	none	none	-
βLG	glycolaldehyde	x-linked	139 nM
S100B	none	none	8.3 μM

Figure 3. Biacore association–dissociation curves of GA-modified bLG. Each line represents the association–dissociation curve for one bLG-GA concentration applied to the flow cell. The table below the figure lists the affinity constants of bLG-GA and the RAGE ligand S100B determined in this experiment.

that five different batches of GA-modified bLG resulted in very different induction levels of TNF-α (as well as IL-6 and IL-8, data not shown). Some batches did not stimulate TNF-α expression (batches 1 and 5), whereas others resulted in intermediate (batch 2) or very high TNF-α induction (batches 3 and 4). This TNF-α stimulation was independent of the degree of modification as shown in Fig. 4B. Batches 1 and 2 were prepared with a 60-fold molar excess of GA relative to bLG resulting in highly cross-linked protein complexes, whereas batch 3 was moderately modified with a tenfold molar excess of GA relative to bLG and batches 4, 5 and 6 (used in Figs. 5 and 8) were only lightly modified with a molar GA:bLG ratio of 1:1. The respective lysine loss, determined by LC-MS/MS [28], was 80–90% for batches 1 and 2, 30% for batch 3 and 3–10% for batches 4, 5 and 6. Between 1 and 6% of the lost lysine was accounted for by the appearance of CML, whereas pyrraline and fructoselysine were not detectable (data not shown). Other modifications, including crosslinks (such as pentosidine), were not analyzed. Overall, it can be concluded that the activation of inflammatory signaling was independent of the extent of lysine modification.

In some experiments, we found that even certain batches of unmodified control bLG resulted in very high TNF-α expression (Fig. 5). Because endotoxin contaminations are known to be a problem in protein and AGE preparations we tested the control and AGE-modified proteins for endotoxin

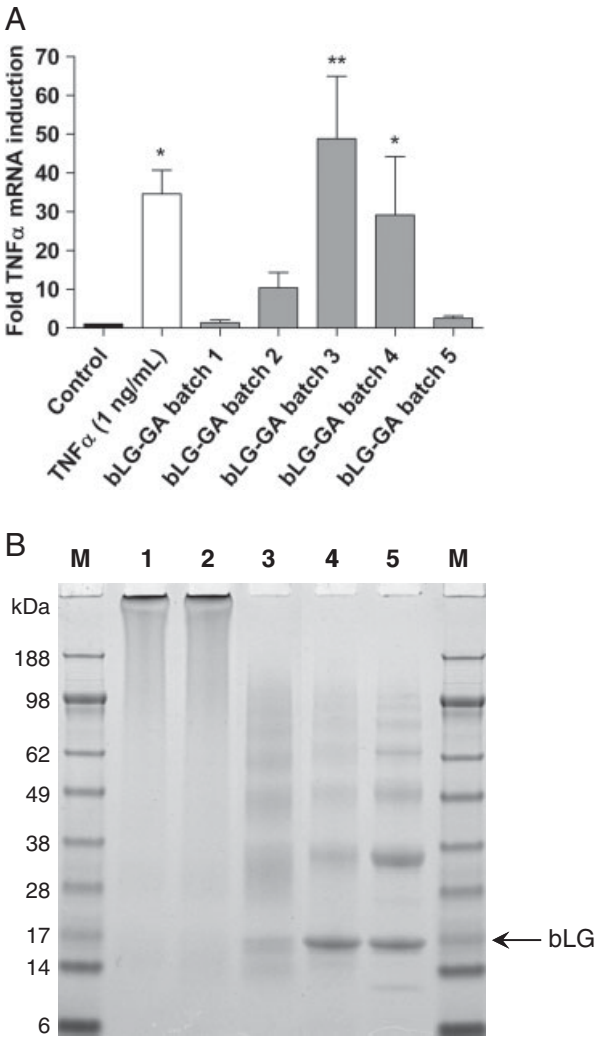
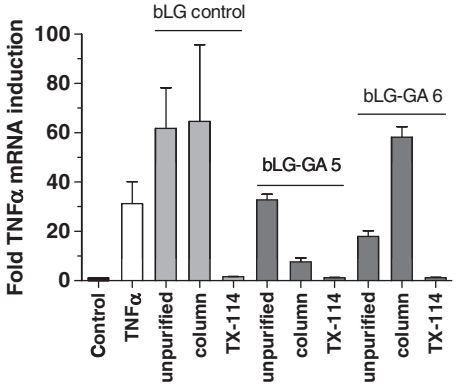


Figure 4. (A) Induction of TNF-α mRNA expression induced by different bLG-GA preparations. The composition of the different batches in described in the text. Asterisks indicate statistically significant differences relative to control at $^{*}p<0.05$, $^{**}p<0.01$. (B) Representative SDS-PAGE of the bLG-GA preparations used. Each lane represents 10 μg of glycated protein.

contamination. Endotoxin was detected in all preparations. To remove endotoxin, we used two methods. The first employed DetoxiGel affinity columns to remove endotoxin as described by the manufacturer. The samples were passed three times over the columns and the final efflux tested negative in the PyrogenT gel clot test. The second method employed the phase partitioning of TX-114 to separate hydrophilic and lipophilic molecules in the aqueous and detergent phase, respectively [21, 22, 25]. Samples were extracted three to five times with 1% TX-114. After five cycles of extraction, the samples tested negative in the PyrogenT gel clot assay but after three extractions, occasionally low levels of endotoxin could still be detected.



Remaining endotoxin	++	-	+-	++	-	+-	++	-	+-
Protein conc. [mg/mL]	73	43	80	16	2	19	19	3	24

Figure 5. Induction of TNF-α mRNA expression by bLG and bLG-GA preparations before and after column- or TX-114 purification using 10 μM protein. The table below the figure shows qualitatively the endotoxin levels remaining after purification determined with the PyrogenT gel clot assay. The protein concentration of the preparation after purification is shown beneath.

Column purification of the bLG-GA samples resulted in a significant loss of protein (Fig. 5), whereas TX-114 extraction generally resulted in a slightly higher protein concentration due to a concentration effect of the extraction procedure. Figure 5 shows that column-purified control bLG and bLG-GA retained their ability to stimulate TNF-α expression in Beas2b cells. However, TX-114 extraction completely abolished the cell-stimulatory activity initially present in these preparations. The variable cell stimulation observed in the two column-purified bLG-GA preparations may be explained by the very low protein recovery after column purification (Fig. 5). These results show that passing AGE preparations over DetoxiGel or similar columns is not sufficient to remove the cell-activating activity present in these preparations despite the fact that no endotoxin could be detected. On the other hand, TX-114 extraction of AGE preparations completely removed all cell-activating activity initially present in the AGE preparations despite the fact that occasionally some endotoxin may still be detectable after extraction. Thus, our data show that purified AGEs by themselves are unable to induce cytokine expression in Beas2b cells. In addition, they indicate that column purification is insufficient to remove the cell-activating contamination.

After TX-114 extraction, small amounts of detergent may remain in the aqueous fraction. To exclude the possibility that TX-114 would interfere with cytokine expression, altered protein folding and/or ligand/receptor interactions, TX-114 was added to the incubation mixes containing TNF-α, unpurified bLG or unpurified GA-bLG at dilutions of 10^{-5} and 10^{-6}. Higher concentrations of TX-114 were toxic for the cells. Figure 6 shows that the addition of

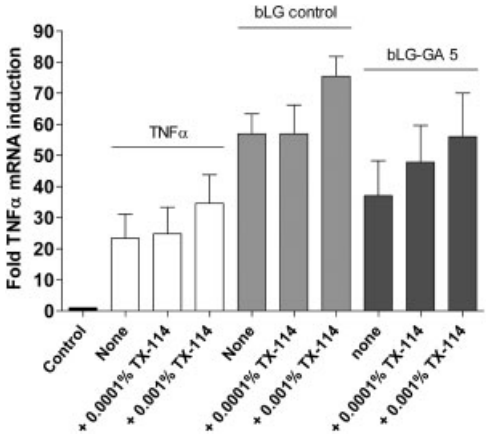


Figure 6. TX-114 does not interfere with cell activation. Beas2b cells were stimulated with TNF-α (10 ng/mL), bLG control and bLG-GA (10 μM each) in the absence or presence of 10^{-5} or 10^{-6} TX-114. Results are of three independent experiments done in triplicate.

TX-114 did not influence the TNF-α expression induced by TNF-α, unpurified bLG and unpurified bLG-GA. In fact, higher TX-114 concentrations might slightly super-stimulate the TNF-α gene expression, although this induction was not statistically significant.

To confirm that TX-114 extraction was indeed able to remove a cytokine inducing activity from AGE preparations, a dilution of the detergent extract was added back to the cells as shown in Fig. 7. The TX-114 detergent extract after purification of control bLG and a bLG-GA preparation was diluted 100 000-fold in culture medium, added back to Beas2b cells and incubated for 1 h prior to analysis of cytokine mRNA expression. The data show that the cell-activating activity was present in the TX-114 detergent extract. More concentrated TX-114 extracts (10^{-4}) were also tested but were found to be toxic to the cells, higher dilutions (10^{-6}) did not result in a significant TNF-α stimulation.

Finally, we also tested the column and TX-114 purified AGEs on another (low) RAGE expressing human cell line, the retinal pigment epithelial cell line ARPE-19. The fold stimulation was lower, but very similar results were obtained as with the Beas2b cells; column purification was not able to remove the cell-activating activity present in the AGE preparations, whereas TX-114 extraction completely removed this activity (Fig. 8).

4 Discussion

Our data show that different batches of GA-modified bLG AGE preparations that possess the capacity to bind to RAGE have very variable capacity to stimulate inflammatory signaling in two human cell lines and that this stimulation was independent of their degree of lysine modification. Even some batches of unglycated commercial proteins stimulated

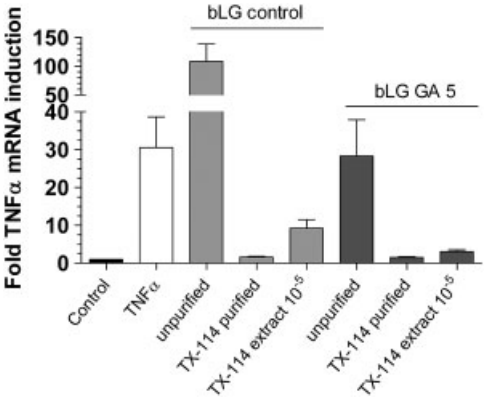


Figure 7. A cell-activating activity is present in the TX-114 extract. A bLG control protein and a bLG modified with GA were extracted with TX-114 as described in Section 2. The resulting purified protein was unable to induce TNF-α mRNA expression. A 10^{-5} dilution of the TX-114 detergent phase after extraction was able to significantly stimulate TNF-α mRNA expression.

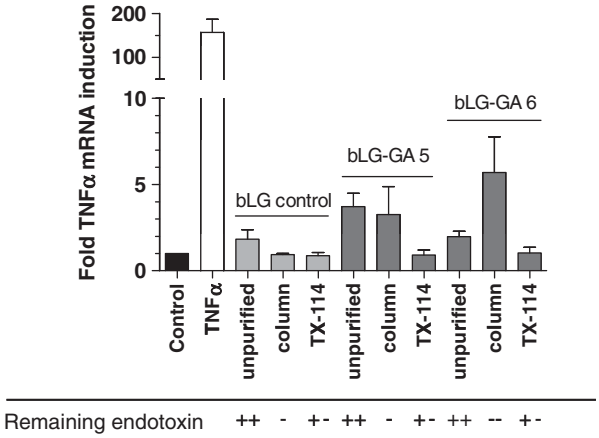


Figure 8. Induction of TNF-α mRNA expression of unpurified and purified control and bLG-GA preparations (10 μM) in human ARPE-19 cells. The same conditions and preparations as shown in Fig. 6 were used. The panel below the graph shows the endotoxin levels determined with the PyrogenT gel clot assay.

inflammatory cell signaling. Further tests revealed that all preparations were endotoxin contaminated to some degree and endotoxin is known to stimulate very similar signaling pathways as AGEs. Passing AGE preparations over Detoxi-Gel columns successfully removed detectable endotoxin but inflammatory signaling persisted. This would normally be taken as evidence that cell activation was due to the AGEs themselves and not to endotoxin. However, the fact that a column-purified, unglycated control bLG protein also resulted in very high cytokine gene induction even after column purification does not fit this scheme. By employing an alternative method of endotoxin removal with TX-114 [21, 22, 25], we demonstrate that the purified control bLG but also all GA-modified AGE preparations were no longer able to stimulate cell activation. The presence of the cell-activating activity was demonstrated in the detergent phase after the purification of contaminated preparations, thus indicating the presence of an extractable lipophilic contamination that was, however, not further characterized. Together, these data show that purified AGEs are unable to activate inflammatory signaling by themselves in the two cell lines investigated.

The health concerns of AGEs may primarily be attributed to browned, cross-linked proteins and we chose GA to modify bLG because this dicarbonyl rapidly and efficiently generates colored, fluorescent and crosslinked proteins. We show that these GA-modified proteins bind to RAGE with an affinity comparable to the known RAGE ligand S100B. We attempted to characterize the modifications introduced by GA but could only detect small quantities of CML but no pyrraline or fructoselysine. We did not analyze for other modifications as our analytical method is restricted to the detection and quantification of these modified amino acids [28]. Spanneberg *et al.* [31] recently described the glycation of gelatin by GA. Similar to our findings, these authors detected only minor amounts of CML but no glyoxal-derived lysine dimer or imidazolinone. Approximately 90% of the lysine modifications could not be accounted for. In addition, these authors showed that lysine was preferentially modified by GA as there was much less loss of arginine residues [31]. In our study, we did not analyze arginine because bLG contains only three arginine residues *per* molecule, whereas there are 16 lysine residues.

There is a large body of data showing that AGEs bind to RAGE and activate cellular signaling resulting in the release of inflammatory mediators such as TNF-α or IL-6. However, since the work of Reznikov *et al.* [20] showed that in the strict absence of endotoxin AGEs are not able to simulate cytokine production in peripheral blood monocytes of healthy donors, it became clear that endotoxin contamination represents a serious problem in cellular AGE research. Since then, most scientists purify their AGE preparations using DetoxiGel or similar columns and, in general, report undetectable endotoxin levels after purification. Our results now show that these columns are inadequate to remove the cell-activating contamination from AGE preparations. Only the alternative method using TX-114 extraction is able to successfully remove this lipophilic cell-activating contamination. In fact, successful extraction of this activity did not always remove all measurable endotoxin since after three extraction cycles low levels of endotoxin could occasionally still be detected. Despite the presence of small quantities of endotoxin, these preparations were completely inactive with respect to cell activation. The fact that no induction of cytokine expression could be observed after TX-114 extraction despite the presence of low but detectable endotoxin levels suggests that these lipophilic contaminations are distinct from endotoxins that are detected by the widely used commercial kit used by us. However, the nature of this lipophilic contamination remains elusive.

There is precedence that AGEs are unable to activate cells under controlled conditions. In addition to the work by Reznikov *et al.* [20], Valencia *et al.* [18, 19] have demonstrated that BSA modified by different reducing sugars (glucose, fructose and ribose) or chemically with GA resulted in AGEs that possess RAGE binding properties. The key message of their studies was that binding to RAGE was not sufficient to trigger cell activation since these modified AGEs were unable to simulate the expression of TNF-α and VCAM1 in endothelial cells. Here, we confirm the findings of Valencia *et al.* [18, 19] that a GA-modified protein can bind to RAGE but that this binding is not sufficient to activate inflammatory signaling.

Cumulatively, the data of Reznikov *et al.* [20], Valencia *et al.* [18, 19] and our data presented here show that AGEs that are endotoxin free or free of certain lipophilic contaminations (our study) are unable to stimulate inflammatory signaling in various cell types (monocytes, endothelial cells, lung and retinal epithelial cells). Thus, it can be concluded that AGEs can only stimulate inflammatory signaling under specific conditions such as the presence of endotoxin or perhaps transition metals [32] but not by themselves. Our data add to this with the demonstration that the commonly used endotoxin removal resins are insufficient to remove the cytokine inducing activity present in most AGE preparations and even some unglycated control proteins. The consequence of this surprising outcome is that probably most data apparently showing that AGEs simulate cells after purification with endotoxin removal resins may not be reliable because the columns generally used are probably not adequate to remove the lipophilic contaminations responsible for this activation.

These results show that binding of purified AGEs to RAGE does not result in biological activity because they are unable to elicit a cellular response by themselves. This opens the way to speculate that because of their high affinity for RAGE, AGEs might act as receptor antagonists because they may compete for the binding of natural RAGE ligands.

In conclusion, our results demonstrate that AGEs purified by TX-114 extraction are unable to stimulate cytokine expression in two human RAGE-expressing cell lines and that the commonly used endotoxin removal columns are unable to remove cell-activating lipophilic contaminations.

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