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Induction of reactive oxygen species and cell survival in the presence of advanced glycation end products and similar structures ☆

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Abstract

Advanced glycation end products (AGEs) that arise from the reaction of sugars with protein side chains and the terminal amino group are supposed to be involved in the pathogenesis of several diseases and therefore the effects of AGEs on cells are the objective of numerous investigations. The effects of AGEs on cells are commonly assumed to be transduced via the *receptor for AGEs* (RAGE) but there are also other receptors known to interact with AGEs and they are likely to be involved in signal transduction. The primary cellular effect of AGEs on cultured cells was found to be the formation of *reactive oxygen species* (ROS). For the present study one murine and three human cell lines were used. The effects of a set of different highly modified AGEs and AGE-like compounds derived from the incubation of different modifiers with BSA were tested for their effects on these cells. Almost all AGEs tested induced the production of *reactive oxygen species* (ROS) in the different cell lines although the intensity of the detected signals varied considerably between the cell lines and are strongly dependent on the AGE used for cell activation. The most highly modified BSA-species were shown to inhibit cell growth in all cell lines, whereas a moderately modified glucose derived BSA-AGE and BSA-GA_{red} did not show any inhibitory effect on cell growth even when a high ROS formation was detected.

Keywords: Advanced glycation end product; AGEs; Reactive oxygen species; ROS; Growth inhibition

1. Introduction

Advanced glycation end products (AGEs) arise from a complex nonenzymatic multi-step reaction of reducing sugars with proteins, namely their amino acid side chains and the terminal amino group. *In vivo*, numerous proteins, particularly long-lived proteins, are found to be AGE-modified, although only to a minimal degree. In cell culture studies *in vitro* prepared AGEs were found to induce cellular activation associated with chemotaxis, oxidative stress and cell proliferation or programmed cell death [1], and *in vivo* AGEs are suspected to be involved in the pathogenesis of several diseases such as chronic clinical complications of diabetes [2], renal failure [2], and Alzheimer's disease [3]. Although other receptors or proteins are known to interact with AGEs many effects of AGEs on cells are commonly attributed to the interaction of the modified protein with the *receptor for AGEs* (RAGE). RAGE is a transmembrane protein of the immunoglobulin superfamily with a molecular mass of 45 kDa that was demonstrated to be present in several cell types such as endothelial cells, monocytes, microglia, and neurons [4]. The AGE binding to RAGE leads primarily to the formation of *reactive oxygen species* (ROS) and hence induces processes related to cellular activation and inflammation [5] such as an activation of $p21^{ras}$, MAP kinases and NF-κB [4]. However, other receptors interacting with AGEs were previously described [4,6] and there is also evidence for the existence of other, currently unidentified proteins interacting with AGEs [7] and at least some of these proteins are likely to contribute to the generation of AGE-induced cellular effects. The induction of ROS as the primary cellular response to AGE-exposure was demonstrated in several cell lines such as rat white adipocytes [8], rat mesangial cells [9],

Abbreviations: AGEs, advanced glycation end products; BSA, bovine serum albumin; CTB, CellTiter Blue; FCS, fetal calf serum; GA, glyoxylic acid; Glc, glucose; HBSS, Hank's balanced salt solution; H_2DCF-DA, dichlorodihydrofluorescein diacetate; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MG, methyl glyoxal; PBS, phosphate buffered saline; RAGE, receptor for AGESs; ROS, reactive oxygen species

☆ This article is dedicated to our dear friend, colleague, and teacher Dr. Jovana Gasic-Milenkovic, who died from cancer in July 2005.

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HUVEC [10], or murine monocytes [11], and therefore intracellular ROS formation is a well suited indicator for assaying the occurrence of AGE-induced effects on cultured cells. The effects of AGEs on cell proliferation was also previously described, but with very different results. Glyceraldehyde modified BSA and casein are found to inhibit cell growth of human promyelocytic leukaemia cells [12]. Contrarily, a growth stimulation was found in several studies after treatment with AGEs. Glucose derived BSA-AGEs were found to stimulate the proliferation of bovine retinal endothelial cells [13] and glyceraldehyde and glycolaldehyde AGEs enhanced the proliferation of human melanoma cells while no or only minor effects were found with glucose and methyl glyoxal derived AGEs [14]. However, AGE-derived cellular effects are strongly dependent not only on the kind of modifier used for AGE-formation but also on the conditions of incubation and finally on the resulting degree of modification and the presence of special structures within the modified protein.

For the present study BSA was modified using different concentrations of glucose (Glc) and methyl glyoxal (MG) as well as one concentration of glyoxylic acid (GA) under reducing conditions. The resulting AGEs were moderately to highly modified. Although by the definition given above only Glc-modified proteins are real AGEs, all modified proteins are referred to as AGEs throughout the text.

The AGEs used in the study were characterized and tested for their effects in cell culture concerning the formation of ROS and the cell growth in the presence of AGEs. Four different cell lines were used for the study. It was found that highly modified AGEs derived from the incubation of BSA with Glc or MG inhibit cell growth and induce the formation of ROS in all cell lines. GA-modified BSA did cause a significant formation of ROS, especially in brain cell lines, but did not inhibit cell growth.

2. Material and methods

2.1. Materials

Chemicals were purchased from the following suppliers: Sigma-Aldrich (Germany): BSA, EDTA, glyoxal, methyl glyoxal, and dichlorodihydrofluorescein diacetate (H^2DCF-DA); Roth (Germany): Roti-Block, and components for PBS; Pierce, (Germany): ECL-kit and BCA reagent 1 and 2; DAKO (Denmark): anti-mouse antibody-horseradish peroxidase conjugate; Fluka (Germany): 2,4-dinitrophenyl hydrazine (2,4-DNPH), 9,10-phenanthrenequinone, and $NaCNBH_3$; Amersham Biosciences (Germany): Rainbow marker 756; Merck (Germany): glucose monohydrate, and glyoxylic acid monohydrate; Promega (Germany): CellTiter Blue reagent; GibcoBRL (Germany) Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), and all other components for the cell culture.

2.2. Preparations and characterization of the modified proteins

AGE modified BSA was prepared by incubating 4 mg/ml BSA in 0.5 M phosphate buffer, pH 8.0 containing 1 mM NaN_3 and 1 mM EDTA with:

500 mM glucose, 6 weeks, 37 °C (BSA-Glc 500)

100, 200, and 500 mM glucose, 4 weeks, 50 °C (BSA-Glc 100, 50 °C; 200, 50 °C or 500, 50 °C)

10, 20, 50 mM methyl glyoxal, 4 weeks, 37 °C (BSA-MG 10, 20, or 50) under sterile conditions. All glassware and the buffer were autoclaved prior to use to inactivate proteases. BSA incubated under the same conditions but without added modifier was used as control. BSA-GA_{red} was prepared as described previously [15] by incubating 4 mg/ml BSA with 3 mM glyoxylic acid in the presence of 0.9 mM $NaCNBH_3$ in 0.5 M phosphate buffer for 24 h at 37 °C. BSA incubated in the presence of $NaCNBH_3$ but without glyoxylic acid was used as control. After the incubation, protein solutions were extensively dialysed against 10 mM phosphate buffer, pH 7.4 and stored at −20 °C. Protein concentrations were determined using the BCA assay according to the supplier's protocol.

Characterization procedures were performed as previously described in detail [16]. Briefly, the number of reacted lysine residues was determined using fluorescamine. Reacted arginine was assayed using 9,10-phenanthrenequinone as described previously [16,17]. The CML content was determined by CML-ELISA kit (kindly provided by Roche Diagnostics, Penzberg, Germany) according to the supplier's protocol and the carbonyl content was assayed using 2,4-DNPH as previously described [16,18]. AGE-absorbance was read at 360 nm and AGE-specific fluorescence was detected at excitation/emission wavelength of 330/395 nm, 365/440 nm, and 485/530 nm at a protein concentration of 1 mg/ml [16]. Additionally, changes in intrinsic protein fluorescence were detected at excitation/emission wavelength of 280/350 nm [16]. All data are given normalized to the corresponding control. Freshly prepared BSA solution was used as an additional control in all cases.

The masses of the non-modified and modified proteins were detected by MALDI-TOF MS as described previously [19].

2.3. Cell lines and cell culture

The following cell lines were used for the study: CaCo-2, a human colonic adenocarcinoma cell line (obtained from DSMZ, Germany); N11 an immortalized murine microglial cell line (kindly provided by Dr. Paola Ricciardi-Castagnoli, Centre of Cytopharmacology, Milan, Italy); U373 MG, a human astrocytoma cell line (kindly provided by Dr. Michael Hüll, University of Freiburg, Germany); and CHME-5, a human embryonic microglial cell line (kindly provided by Prof. Pierre Talbot, University of Quebec, Canada). Cells were grown in cell culture flasks (25 cm^2, Greiner) in DMEM supplemented with FCS (10% for N11, U373 MG and CHME-5, 20% for CaCo-2), glutamine, non-essential amino acids (for CaCo-2) and penicillin/streptomycin at 37 °C, 5% CO_2 and 95% humidity and harvested with Trypsin (2 min for N11, U373 MG and CHME-5, 10 min for CaCo-2). Suspended cells were counted in a CASY 1 cell counter (Schärfe, Germany) for experiments.

2.4. Detection of RAGE using Western blot analysis

Samples were obtained from cells that were washed twice with PBS before lysed in Laemmli sample buffer [20] without added bromphenol blue. The protein concentrations were adjusted by measuring the absorbance of the cell lysate at 280 nm. SDS-PAGE was performed with equal amounts of protein for each cell line, according to the procedure of Laemmli [20] using 12% acrylamide gels in a Mini-PROTEAN 3 chamber (BIO-RAD). Rainbow marker 756 was used as molecular weight marker. Proteins from unstained SDS-PAGE gels were blotted onto a nitrocellulose membrane for 90 min at 150 A in a Semi-dry blotting system (C.B.S. Scientific. Co, USA) using a buffer containing 25 mM Tris and 190 mM glycine, pH 8.3 as transfer buffer. Following an 1 h blocking with 10% Roti-Block the membrane was incubated for 1 h with a monoclonal mouse-anti-RAGE antibody (kindly provided by Dr. Th. Henle and Dr. Bernd Weigele, TU Dresden, Germany) in Tris buffered saline (TBS) containing 10% Roti-Block. The primary antibody was removed by washing the membrane four times with TBS. Afterwards the blot was incubated for 1 h with a monoclonal anti-mouse IgG conjugated with horseradish peroxidase in TBS containing 10% Roti-Block. Unbound antibody was removed by washing the membranes four times with TBS containing 0.05% Tween 20. Bound antibody was detected using the ECL-assay according to the supplier's protocol.

2.5. CellTiter Blue cell survival assay

Cell growth in the presence of AGEs was assayed using the CellTiter Blue cell viability assay. 10000 cells in case of CaCo-2 and 5000 cells for the brain cell lines were seeded into 96-well cell culture plates (TPP, Switzerland) in 100 μl medium and 50 μl of the protein sample (final concentration of protein sample 15 μM). The cells

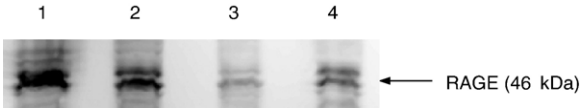

Fig. 1. Western blot image of the detection of RAGE in the lysates of CaCo-2 (lane 1), CHME-5 (lane 2), N11 (lane 3), and U373 MG (lane 4). Cells were lysed in Laemmli buffer (without bromphenol blue) and the protein concentrations in the cell lysates were adjusted by measuring the absorbance at 280 nm.

were grown for 48 h and the medium was removed from the wells. 1.5 ml of CellTiter Blue reagent were mixed with 8.5 ml fresh medium and 100 µl of the solution were added to the cells. The brain cell lines were incubated for 1.5 h and CaCo-2 for 3 h before fluorescence was detected at excitation/emission wavelengths of 540/590 nm in a FLUO-STAR plate reader (BMG, Germany).

2.6. Reactive oxygen species assay

The AGE-induced formation of reactive oxygen species was measured using dichlorodihydrofluorescein diacetate (H_2DCFDA) as described previously [11] with some modifications. 10000 cells per well were seeded in a total volume of 100 µl medium (as described in the cell culture paragraph) in 96-well cell culture plates (TPP, Switzerland) and grown for 48 h (CaCo-2) or 24 h (brain cell lines). The medium was removed and each well was washed with 150 µl HBSS before loading the cells with H2DCF-DA (100 µl, 0.1 mM in HBSS) for 1 h. The dye reagent was removed and the wells were washed with 150 µl HBSS. Samples were added in a total volume of 100 µl with a protein concentration of 10 µM in HBSS. Freshly prepared BSA-solution was used as control. After a 20 h incubation fluorescence was read at excitation/emission wavelengths of 485/520 nm in a FLUO-Star reader (BMG, Germany). Wells containing cells and protein but no dye were used to correct the detected signals for the AGE fluorescence.

2.7. Statistics

All data given in the text represent the averages of three independent experiments with at least 3 data-points. All values are given normalized to the value obtained with non-incubated BSA-solution.

The data were compared to the data obtained with non-incubated BSA-solution by Student's t test. Significances are given as * (P=0.01–0.05), ** (P=0.001–0.01), and *** (P<0.001).

3. Results

3.1. Presence of RAGE in the cell lines

The presence of RAGE in the cell lines used in the study was probed by Western blot using a monoclonal antiRAGE antibody. As shown in Fig. 1 RAGE was found to be present in all cell lines used in the study. The results for N11 and CaCo-2 cells are consistent with previous studies, where RAGE was demonstrated in CaCo-2 by RT-PCR and Western blot [21] and in N11 by Western blot [22]. According to the literature CHME-5 and U373 MG cells were not previously tested for the receptor for AGEs. The presence of RAGE in the cell lines used is assumed to prove the general ability of the cells to interact with AGEs and should not imply that all effects described in the present study are necessarily mediated by RAGE.

3.2. Characterization of the AGEs used in the study

The characterization data of all modified proteins used in the study are given in Table 1 (molecular masses and side chain modifications) and Table 2 (spectroscopic data). The AGEs were prepared at 37 °C unless otherwise stated. The BSA-AGEs derived from the incubation with Glc at 50 °C and with MG show the highest degrees of modification although the degree of arginine modification is higher for MG than for Glc derived modified BSA. Accessible CML groups are found in all modified proteins. The highest numbers of CML-modifications were detected in the BSA-AGEs produced at 50 °C, while the CML-content of the proteins modified with MG is relatively low. However, even BSA-GA_{red}, that was prepared in accordance to a protocol that is assumed to result mainly in the formation of CML-modifications [15], contains only 0.8 mol accessible CML per mol BSA. The carbonyl content, which is a

Table 1
Data for AGA-characterization for the AGEs used in the study

	MW [Da]	Lysine %mod.	Arginine % mod.	mol CML/ mol BSA	mol carbonyl/ mol BSA
BSA	66553			0	0
BSA-Glc 500	71582	69	30	1.14	3.3
control BSA-Glc 500	66475	0	0	0	0
BSA-MG 10	71808	59	67	0.09	4.4
BSA-MG 20	72177	82	78	0.41	7.1
BSA-MG 50	76484	96	88	0.95	12.0
control BSA-MG 10–50	66562	0	0	0	0
BSA-Glc 100, 50 °C	70673	67	53	4.90	3.8
BSA-Glc 200, 50 °C	72896	86	62	5.13	5.0
BSA-Glc 500, 50 °C	75774	95	73	5.34	7.2
control BSA-Glc 100–500, 50 °C	66478	0	0	0	0
BSA-GA_{red}	67291	35	8	0.80	0
control BSA-GA_{red}	66506	0	0	0	0

The data are given either in % modified compared to the corresponding control (degree of reacted arginine and lysine side chains) or in mol modifications per mol BSA (CML and carbonyl groups).

Table 2
Spectrospic data for the AGEs used in the study

	A [360] AU	Fluo [280/350] % of control	Fluo [330/395] norm. control	Fluo [365/440] norm. control	Fluo [785/530] norm. control
BSA-Glc 500	0.36	10	32	32	34
control BSA-Glc 500	0	100	1	1	1
BSA-MG 10	1.10	1.7	47	40	95
BSA-MG 20	2.10	0.5	24	39	122
BSA-MG 50	3.61	0.0	11	15	123
control BSA-MG 10–50	0	100	1	1	1
BSA-Glc 100, 50 °C	0.91	4.0	19	12	65
BSA-Glc 200, 50 °C	1.50	1.5	18	11	85
BSA-Glc 500, 50 °C	1.55	0.7	14	8	79
control BSA-Glc 100–500, 50 °C	0	100	1	1	1
BSA-GA_{red}	0	94	1	1	1
control BSA-GA_{red}	0	100	1	1	1

The data are given normalized to the corresponding control. For the AGE specific absorbance at 360 nm the absorbance of BSA control at 280 nm was used to normalize the data.

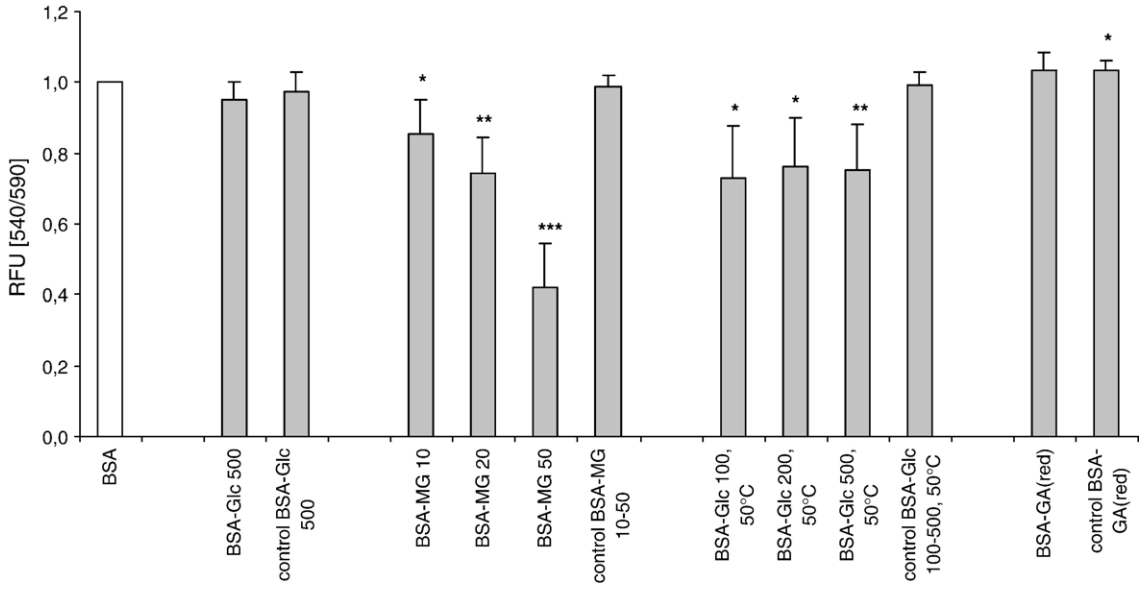

Fig. 2. Cell growth of CaCo-2 cells in the presence of differently modified BSA. Cells were seeded and grown for 48 h in the presence of 15 μM protein sample in standard medium. The cell growth was determined using the CellTiter Blue assay in fresh medium.

good indicator for the formation of stable AGE-structures [23] is highest in 50 mM MG derived BSA-AGE followed by the AGE from in the incubation with 500 mM Glc at 50 °C. No carbonyl groups could be detected in BSA-GA_{red}.

AGE specific absorbance was detected at 360 nm. The given data are normalized to the absorbance at 280 nm of the corresponding control, since non-modified BSA shows no absorbance at 360 nm [16]. Changes in the protein structure were detected using the intrinsic protein fluorescence of tryptophan. The native structure was found to be significantly affected in all modified proteins except BSA-GA_{red}, which shows only a minor decrease in the intrinsic protein fluorescence. AGE specific fluorescence was detected at the wavelengths indicated and was found to be high for all modified proteins except BSA-GA_{red}, which is virtually non-fluorescent. However, compared to fresh BSA even the BSA control incubated at 50 °C without modifier shows significantly affected spectroscopic data (data not shown in Table 2). The intrinsic protein fluorescence was found to be reduced to 70% of the value obtained with fresh BSA and the AGE-specific fluorescences were enhanced by a factor of 6, 12, and 4 at excitation/emission wavelength of 330/395 nm, 365/440 nm, and 485/530 nm when compared to fresh BSA. This is probably a result of the relatively drastic incubation conditions, that are likely to affect the native protein structure and from ongoing AGE-formation processes of already existing glycation-structures within the virtually non-modified protein. The

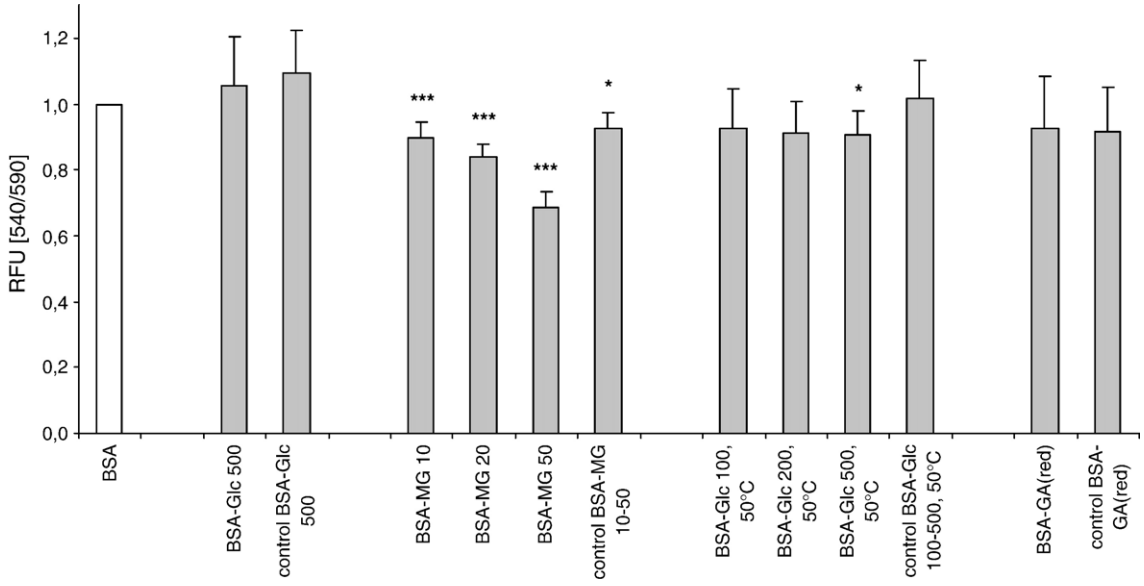

Fig. 3. Cell growth of N11 cells in the presence of differently modified BSA. Cells were seeded and grown for 48 h in the presence of 15 μM protein sample in standard medium. The cell growth was determined using the CellTiter Blue assay in fresh medium.

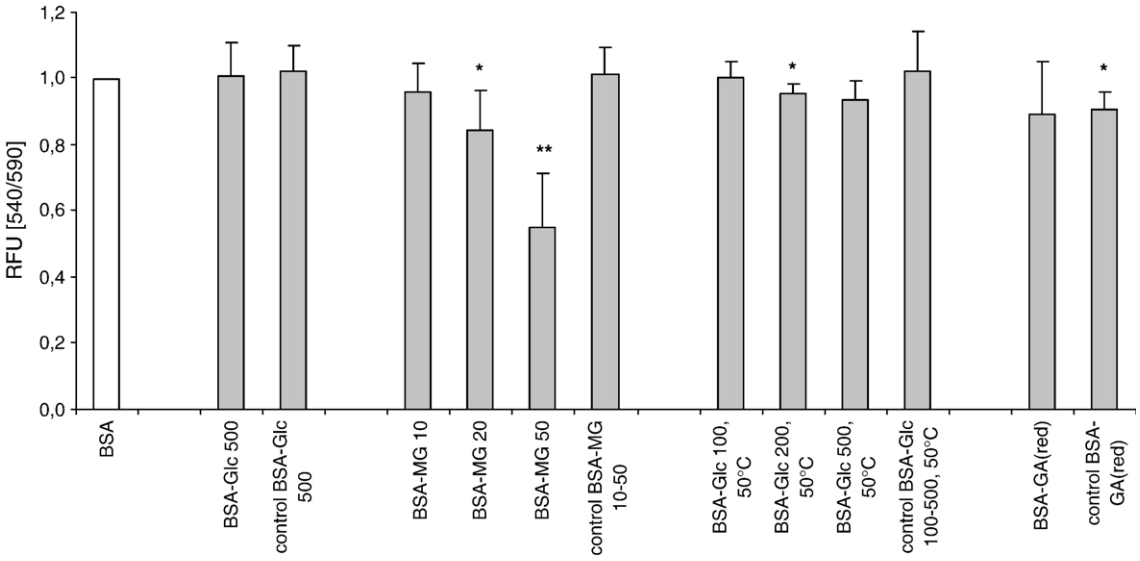

Fig. 4. Cell growth of U373 MG cells in the presence of differently modified BSA. Cells were seeded and grown for 48 h in the presence of 15 μM protein sample in standard medium. The cell growth was determined using the CellTiter Blue assay in fresh medium.

other controls showed only slightly enhanced AGE-fluorescences when compared to fresh BSA.

3.3. Cell growth in the presence of AGEs

The results from the cell growth experiments for all cell lines are shown in Figs. 2–5. The data represent the averages of three individual experiments with at least 3 data-points. All data are given normalized to the signal obtained when using freshly prepared BSA-solution. As obvious from the figures, only the very highly modified BSA-species, especially the MG-derived AGEs, were able to inhibit cell growth at the applied conditions. Neither BSA-AGE derived from the incubation with 500 mM Glc at 37 °C nor the BSA-GA_{red} had any effect on the cell growth of all cell lines used in the study. N11 and U373 MG cells are only slightly growth-inhibited in the presence of BSA-AGEs derived from the incubation with glucose at 50 °C, whereas the cell number of CaCo-2 and CHME-5 is significantly reduced compared to the control when grown in the presence of these AGEs. Interestingly, CHME-5 did even show a relatively strong response on the different degrees of modification with these Glc-modified proteins, which was not found for the other cell lines.

The concentration dependence of the growth inhibiting effect of BSA-MG 50 on CHME-5 is shown in Fig. 6. The growth inhibition was found to be concentration dependent although only slight differences were found in the effects caused by the different AGE-concentrations used.

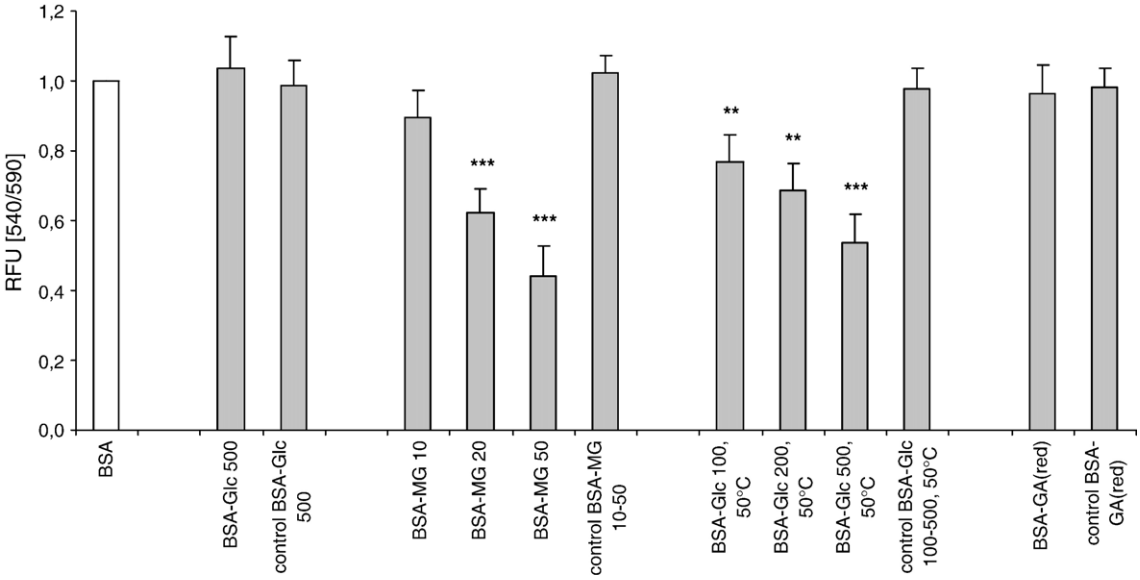

Fig. 5. Cell growth of CHME-5 cells in the presence of differently modified BSA. Cells were seeded and grown for 48 h in the presence of 15 μM protein sample in standard medium. The cell growth was determined using the CellTiter Blue assay in fresh medium.

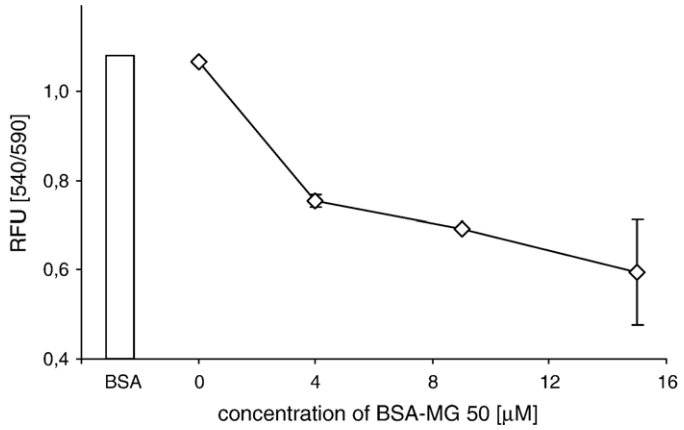

Fig. 6. Cell growth of CHME-5 in the presence of increasing concentrations of BSA-MG 50. Cells were seeded and grown for 48 h in the presence of the indicated sample concentrations in standard medium. The cell growth was determined using the CellTiter Blue assay in fresh medium.

3.4. Formation of reactive oxygen species as a cellular response to AGEs

The results from the ROS-assays for all cell lines are shown in Figs. 7–10. The data represent the averages of three individual experiments with at least 3 data-points. The data were corrected for the intrinsic AGE-fluorescence before evaluation. All data are given normalized to the signal obtained when using freshly prepared BSA-solution. As shown in Figs. 7–10 all cell lines produced ROS in the presence of AGEs, but the efficiency of the different AGE-proteins varied considerably between the cell lines. Remarkable differences are found for GA-modified BSA, which caused a high ROS formation in all brain cell lines, whereas CaCo-2 cells were only slightly stimulated. In U373 MG and CHME-5 cells BSA-GA_{red} caused the highest ROS-signals among all AGEs tested. The control for BSA-GA_{red} did also cause a moderately enhanced ROS-production in the brain cell lines as compared to fresh BSA. This might be an effect caused by the incubation of the protein in the presence of $NaCNBH_3$. The second protein with significantly different cell responses is the BSA-AGE derived from the incubation with 500 mM Glc at 37 °C. While CaCo-2 cells were considerably stimulated by this protein, no or only minor stimulation was detected in all brain cell lines. This is an unexpected finding because the BSA-Glc 500, 37 °C was found to contain 1.14 mol/mol accessible CML groups, which was previously reported to be an important cell affecting modification [24] and should therefore cause at least some stimulation. Thus, other modifications than CML seem to be responsible for the formation of ROS in the brain cell lines. The other AGEs tested induced a high ROS-formation in all cell lines, although CaCo-2 and N11 were more stimulated by these highly modified AGEs than U373 MG and CHME-5 cells. In CaCo-2 and N11 cells a slightly enhanced ROS-formation can

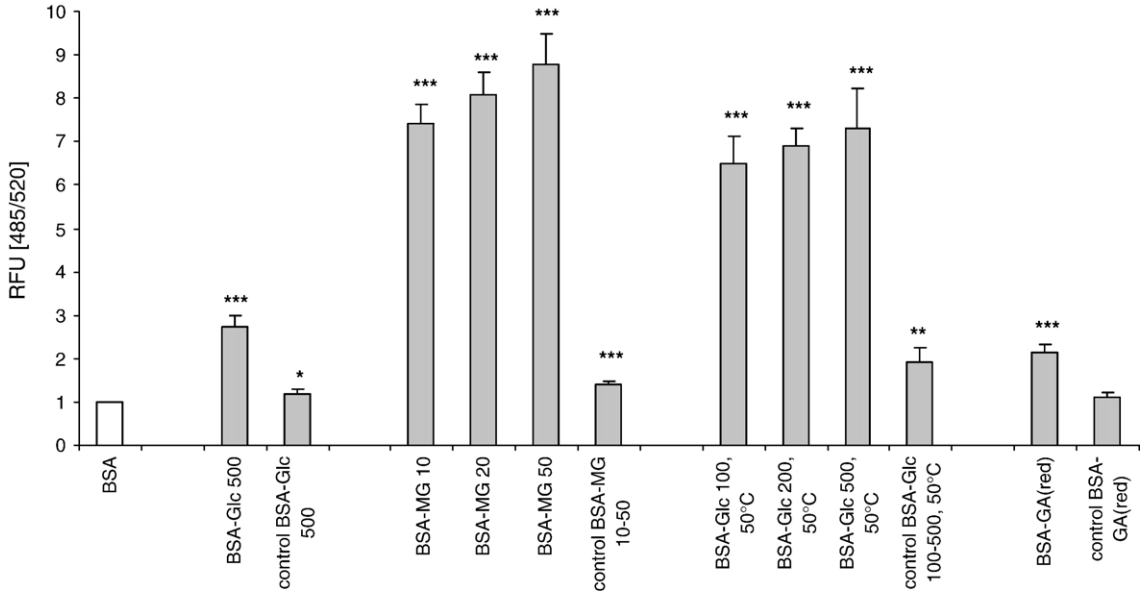

Fig. 7. Data obtained for the stimulation of CaCo-2 cells with differently modified BSA. The stimulation was performed for 20 h with a sample concentration of 10 μM in HBSS. The data are given normalized to the signal obtained with fresh BSA.

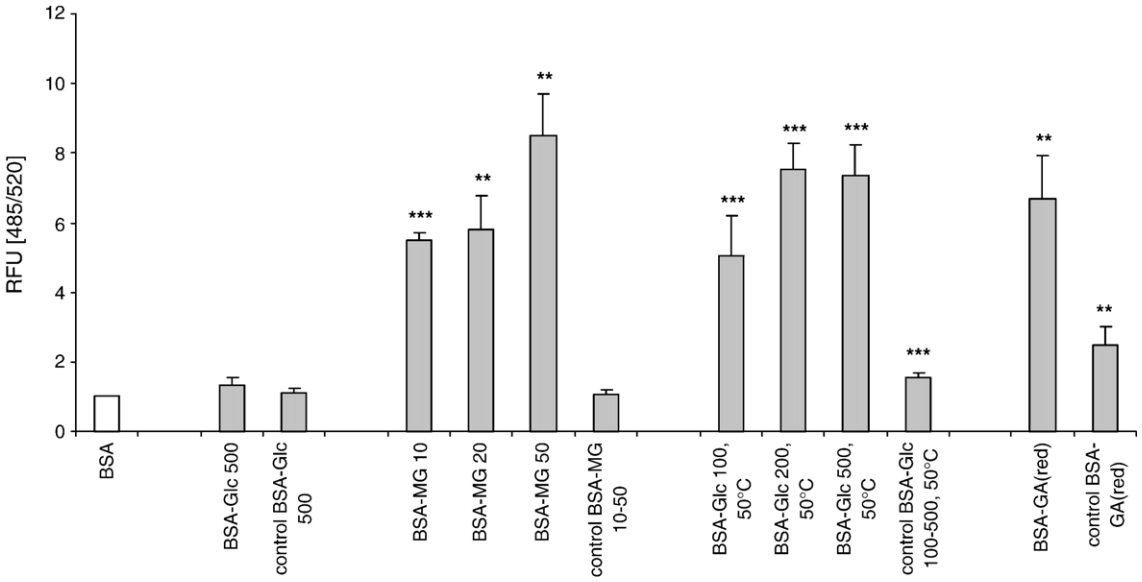

Fig. 8. Data obtained for the stimulation of N11 cells with differently modified BSA. The stimulation was performed for 20 h with a sample concentration of 10 μM in HBSS. The data are given normalized to the signal obtained with fresh BSA.

also be detected for the control for BSA-AGEs incubated at 50 °C.

4. Discussion

As shown above the presence of AGEs derived from incubation of BSA with glucose, methyl glyoxal, or glyoxylic acid (under reducing conditions) under different conditions caused the formation of ROS and inhibited cell growth in the cell lines studied. The proteins used in the present study are highly modified with a degree of modification with at least 35% of lysine and 8% of arginine residues modified. Although the degrees of modification of the AGEs used are non-physiologic it should be mentioned that the complications caused by AGEs *in vivo* are usually a result of long-term exposure of cells to minimally modified AGE-proteins, which might *in vivo* be modified from different sugars or dicarbonyl compounds in a long term process. As cell culture incubation is usually done using very short incubation times and low protein concentrations (when compared to physiological conditions), the use of highly modified AGEs, although they are non-physiologically modified, is assumed to be a valuable model to study the general effectiveness of AGEs in cell damaging processes. In the present study cell growth was most effectively inhibited by MG derived AGEs, whereas a strong ROS formation was observed not only with MG derived AGEs but also with Glc-AGEs prepared at 50 °C, and in the brain cell lines with BSA-GA_{red}.

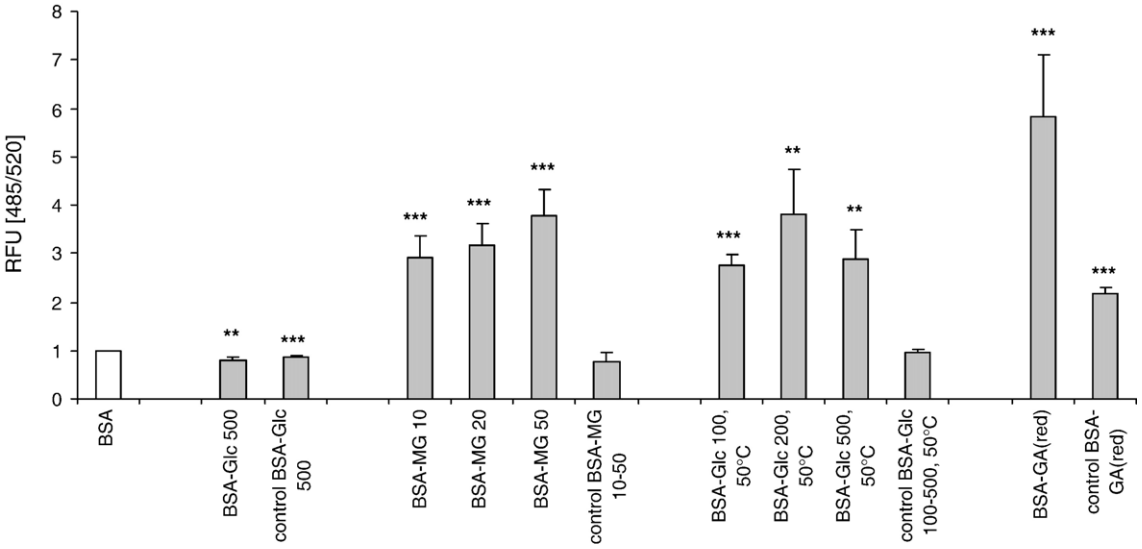

Fig. 9. Data obtained for the stimulation of U373 MG cells with differently modified BSA. The stimulation was performed for 20 h with a sample concentration of 10 μM in HBSS. The data are given normalized to the signal obtained with fresh BSA.

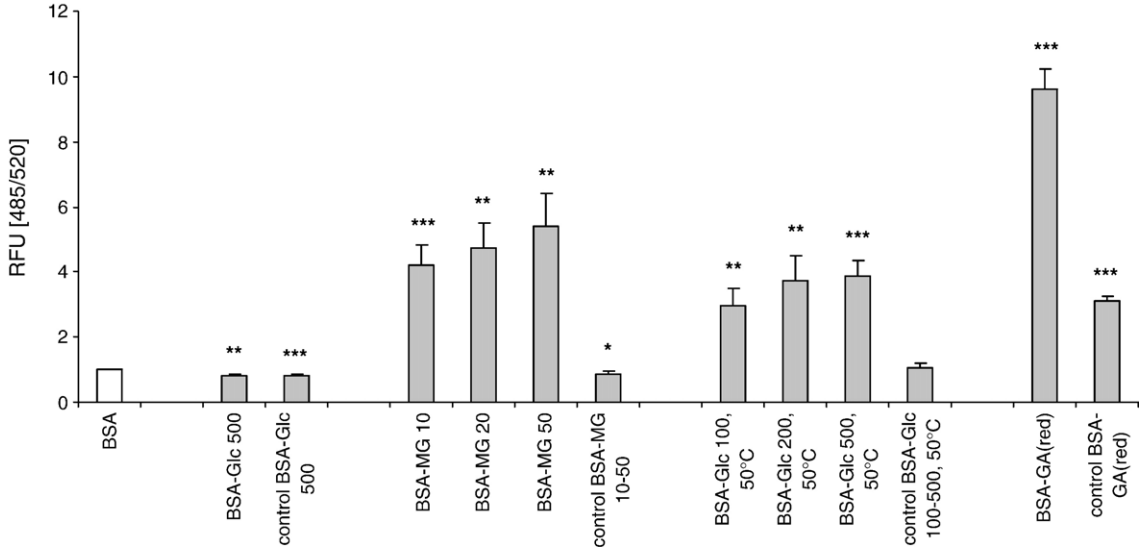

Fig. 10. Data obtained for the stimulation of CHME-5 cells with differently modified BSA. The stimulation was performed for 20 h with a sample concentration of 10 μM in HBSS. The data are given normalized to the signal obtained with fresh BSA.

Although to a different extent all cell lines in the study were found to be growth inhibited by the Glc-AGEs produced at 50 °C and by MG-AGEs. The BSA-Glc 500 produced at 37 °C and BSA-GA_{red} did not show a significant effect on cell growth. Therefore, our results are in contrast to previous findings, where a stimulation of cell proliferation was found after AGE-treatment [13,14] and in agreement with results published by Usui et al., who found a growth inhibiting effect of AGEs [12]. However, the effects of AGEs are strongly dependent not only on the cell lines and the conditions used for the tests, but also on the kind of AGE (modifier, conditions for incubation, resulting degree and kind of modification) and hence, a comparison of data from

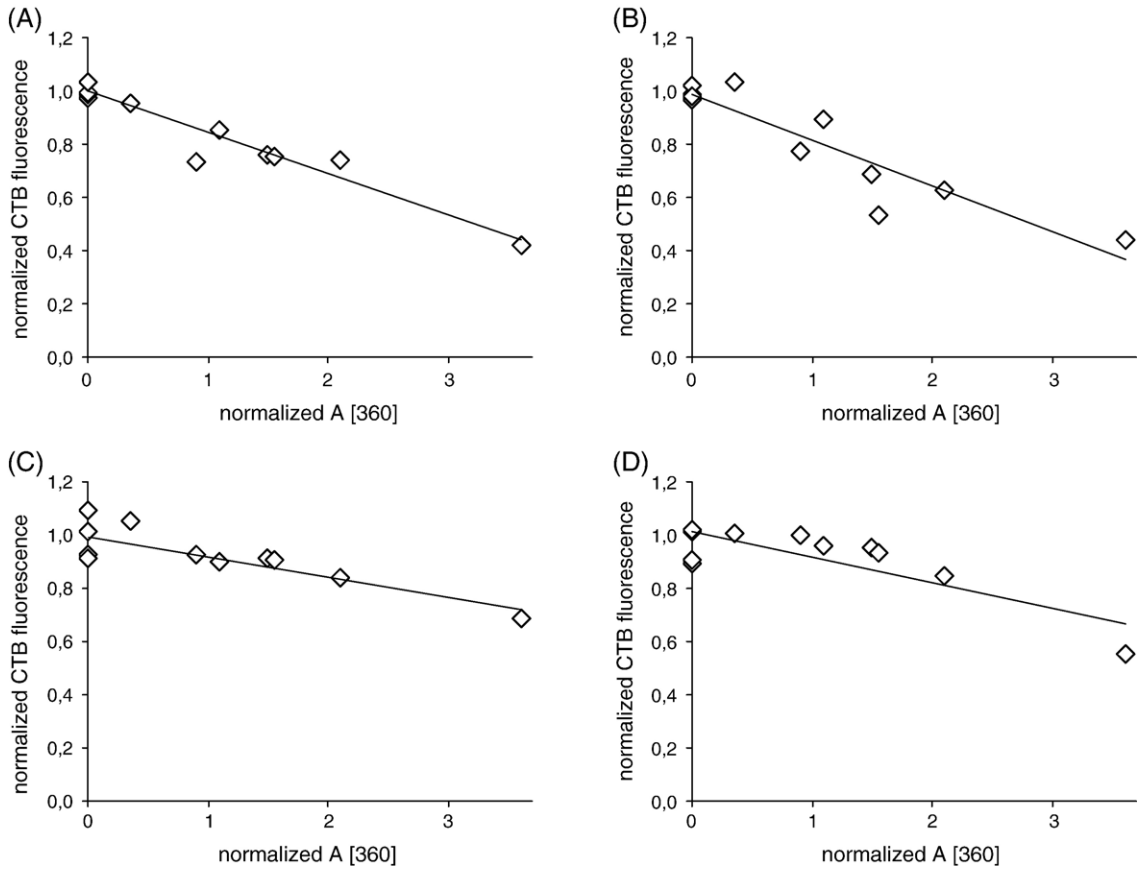

Fig. 11. Correlation of the CTB fluorescence versus the AGE-absorbance at 360 nm for CaCo-2 (A), CHME-5 (B), N11 (C), and U373 MG (D).

different studies without an exact knowledge of all parameters in the test is almost impossible. But when using identical conditions for different cell lines as done in the present study, comparable results were obtained, although the extend of growth inhibition varied from cell line to cell line. However, when searching for the AGE-characteristics, that are correlated to the growth inhibiting effects of the AGEs used in the study, it was found that the decrease in the CTB-fluorescence in AGE-treated cells is linearly correlated to the increase in AGE-absorbance at 360 nm (Fig. 11). This parameter was previously found to be a useful tool for the biochemical characterization of AGEs [16,19] and could now be shown to also correlate with the growth inhibiting effect of AGEs. A similar correlation was found with the molecular mass of the AGEs and the degree of modification, although they were not that exact as the correlation with the A [360] (data not shown). The AGE specific fluorescence or the number of CML or carbonyl groups did not correlate with the growth inhibiting effects of the AGEs.

For the AGE-induced intracellular ROS formation no correlation with AGE-parameters could be found that was valid for all cell lines. For CaCo-2 the formation of ROS was linearly correlated with the degree of arginine modifications (data not shown) but this correlation was not applicable for any of the other cell lines, where BSA-Glc 500 (37 °C) did not cause any ROS formation, while having 30% of arginine groups modified. Contrarily, BSA-GA_{red} had only 8% of arginine groups modified and caused a very high ROS formation in the brain cell lines. However, all AGEs used in the study induced the formation of significant amounts of ROS, except for BSA-Glc 500 (37 °C) in the brain cell lines. This is somewhat unexpected, because this AGE was produced according to a more or less often used standard protocol and similar AGEs were found to induce cellular effects in different studies (e.g. [8,13]). Contrarily, although different cells and different cellular parameters were studied, Valencia et al. found a lack of VCAM1 and TNF-α activating activity for similar AGEs [25], what is in agreement with the lack of growth inhibiting and ROS-formation activity of BSA-Glc 500 (37 °C) observed in the present study.

Interestingly, the formation of ROS seems to be not necessarily correlated to the cytotoxic effects of AGEs. While a high ROS production and growth inhibition was observed with Glc-AGEs (50 °C) and MG-AGEs, BSA-GA_{red} did cause a high formation of ROS, especially in the brain cell lines, without any effect on cell growth. This suggests, that the ROS-formation caused by BSA-GA_{red} might induce a different intracellular signalling pathway than the other AGEs in the study, or that the growth inhibiting effect is ROS-independent cell response caused by modifications, that are not found in BSA-GA_{red} but in the other AGEs.

Most controversial are the data obtained with BSA-GA_{red}, which was prepared in accordance to a protocol that is assumed to result in a more or less specifically CML-modified protein [15,24]. But it was shown in the characterization, that the BSA modified with GA under reducing conditions, which should be CML-modified, most likely contains a high number of modifications other than CML (mass increase of 765 Da compared to the corresponding control, 27% of reactive side chains modified). BSA-GA_{red} did cause a high production of ROS in the brain cell lines and also a moderately increased signal in CaCo-2, whereas there was no growth inhibiting effect detectable in the CTB assay. However, as shown in Table 1, the accessible CML content was found to be much higher in the glucose derived BSA-AGEs than in BSA-GA_{red}. Anyhow, the BSA-Glc 500 (37 °C) did not cause any ROS-formation in the brain cell lines and the ROS-formation in CHME-5 and U373 MG cells caused by the 50 °C Glc-AGEs (accessible CML 4.9–5.3 mol per mol BSA) was significantly lower than the signal with BSA-GA_{red}. Therefore, it can be concluded that either CML is not the modification that is responsible for the high production of ROS, or that the presence of CML alone is not sufficient for the signal transduction pathway leading to growth inhibition. It is also possible, that a combination of CML with another modification serves as the binding motif within the modified protein.

5. Conclusion

In the present study it was shown that exposure of cultured cells to differently prepared AGEs resulted in reduced cell growth and intracellular ROS formation. Different AGEs were shown to cause different intensities in cellular response and the results did also vary from cell line to cell line. Growth inhibition was strongest with MG derived AGEs and could also clearly be demonstrated with Glc-AGEs from incubation at 50 °C. The growth inhibiting effect was found to be in good correlation with the absorbance at 360 nm of the AGE samples used.

Additionally, almost all AGEs in the study did provoke the formation of intracellular ROS, but this effect could not be correlated to any of the parameters from the AGE-characterization. Furthermore, a high ROS-formation is not necessarily related to a strong growth inhibiting effect. This could clearly be shown for BSA-GA_{red}, that did induce a high ROS production in brain cell lines but had no growth inhibiting effect. Therefore, ROS formation and growth inhibition in response to AGE exposure might be transduced by independent or partially independent signalling pathways that might be caused by different modifications or even by different AGE-receptors.

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