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Original Contribution

Oxidative stresses induced by glycoxidized human or bovine serum albumin on human monocytes

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ABSTRACT

Oxidative stress and protein modifications are frequently observed in numerous disease states. Albumin, the major circulating protein in blood, can undergo increased glycoxidation in diabetes. Protein glycoxidation can lead to the formation of advanced glycoxidation end products, which induce various deleterious effects on cells. Herein, we report the effect of glucose or methylglyoxal-induced oxidative modifications on BSA or HSA protein structures and on THP1 monocyte physiology. The occurrence of oxidative modifications was found to be enhanced in glycoxidized BSA and HSA, after determination of their free thiol group content, relative electrophoretic migration, carbonyl content, and antioxidant activities. Cells treated with glycoxidized albumin exhibited an overgeneration of intracellular reactive oxygen species, impairments in proteasomal activities, enhancements in RAGE expression, and an accumulation of carbonylated proteins. These novel observations made in the presence of a range of modified BSA and HSA facilitate the comparison of the glycoxidation extent of albumin with the oxidative stress induced in cultured monocytes. Finally, this study reconfirms the influence of experimental conditions in which AGEs are generated and the concentration levels in experiments designed to mimic pathological conditions.

Introduction

Currently, in the United States alone, more than 17 million people suffer from diabetes and this number may double before 2030 [1,2]. Type 2 diabetes or non-insulin dependent diabetes (NIDD) represents approximately 85% of all cases of diabetes. This disease is a metabolic disorder characterized by hyperglycemia and is dramatically associated with severe complications. NIDD could double the risk of developing cardiovascular disease, which represents the leading cause of mortality in western countries [3].

Albumin is the most abundant serum protein with normal concentrations in the range of 35–50 g/L. This protein possesses a wide range of biological properties [4], and there has been ample evidence suggesting that it exerts significant antioxidant activity [5]. In fact, albumin represents the major and predominant circulating antioxidant molecule in plasma known to be exposed to continuous stress [6–8].

Oxidative modifications of proteins could occur in diabetes, which is one of the important pathological conditions associated with the early occurrence of vascular complications, together with functional alterations of albumin. In NIDD, albumin undergoes increased glycation and glycoxidation [9]. The glycation phenomenon corresponds to the nonenzymatic and nonoxidative covalent attachment of glucose molecule to protein [9]. Glycoxidation refers to the radical-mediated oxidation reaction of both free and protein-bound sugars [2]. Amadori rearrangement of the above glycoxidized protein gives rise to deleterious, advanced glycoxidation (also termed advanced glycation) end products (AGEs) [10,11]. The formation and accumulation of AGEs constitute the characteristic features of tissues in NIDD patients. The pathological conditions of diabetes has been associated with an enhanced oxidative stress [12]. Oxidative stress in diabetes could originate from various processes, such as excessive production of oxygen radicals from the autoxidation of glucose, glycoxidized proteins, and glycoxidation of antioxidative proteins [13]. The compiled data from biochemical, animal and epidemiological studies strongly support the hypothesis that glycoxidative modifications of circulating proteins play a pivotal and causative role in the pathogenesis of NIDD [14–17].

Interaction of AGEs with their receptors (RAGE) induces several cellular phenomena potentially relating to diabetic complications.

Abbreviations: AGEs, advanced glycation (or glycoxidation) end products; ANOVA, analysis of variance; AU, arbitrary unit; BC, bathocuproinedisulfonic acid; BSA, bovine serum albumin; BSA_{GX}, BSA incubated with × mM glucose; BSA_{G0}, BSA incubated in the absence of glucose; CEL, carboxyethyllysine; CML, carboxymethyllysine; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescein diacetate; DCFH, dichlorofluorescein; ECL, enhanced luminol chemiluminescence; FACS, fluorescence-activated cell sorting; HT_{50}, 50% hemolysis time; LDH, lactate deshydrogenase; MALDI, matrix-assisted laser desorption ionization; MGO, methylglyoxal; NIDD, non-insulin-dependent diabetes; PBS, phosphate-buffered saline; PLS, partial least squares; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species.

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AGEs were demonstrated to augment inflammatory responses in monocytes by up-regulating multiple signaling pathways via RAGE, leading to vascular cell dysfunction [18]. Conversely, the AGEs product inhibitor LR-90 has been shown to exert anti-inflammatory effects in human monocytes [19]. AGEs have been identified as one of the factors closely associated with the vascular complications in NIDD. The implication of glycoxidized albumin in the generation of oxidative stress and damage on inflammatory cells should be better specified. We previously reported the effects of oxidative modifications induced by the BSA glycoxidation on its structure, antioxidant properties, and adipose cell physiology [20–22]. The majority of literature in this regard reports the use of bovine serum albumin. Hence it is of utmost importance to compare the effects of glycoxidation on both the structures of human serum albumin (HSA) *and* bovine serum albumin (BSA) and to investigate and compare the effects of such glycoxidized albumin on monocyte cell physiology.

Here, we report the overgeneration of intracellular reactive oxygen species (ROS), impairments in proteasomal activities, accumulation of carbonylated proteins, and enhanced expression of RAGE in glycoxidized albumin-treated cells. These novel observations have been made in the presence of various modified BSA and HSA, which allows a comparison between the extent of albumin glycoxidation and the subsequent induced oxidative stress in cultured monocytes.

Materials and methods

Preparation of AGEs

For the preparation of glycoxidized albumin, nonrecombinant bovine serum albumin (Sigma Cat. No. A2153) and human serum albumin were used. HSA were purified in our laboratory from whole human plasma as follows: isolation of serum albumin from fresh human plasma was based on an extensive dialysis against 50 mM Tris/HCl, pH 7.4, followed by an affinity chromatography using Cibacron Blue 3G linked to agarose (Amersham Cat. No. 17-0948 01) as ligand for albumin. A, 1.5 M NaCl, pH 7.4, buffer was used for the desorption of bound albumin from Cibacron blue–agarose following elution of other plasmatic proteins with a 50 mM Tris/HCl, pH 7.4, buffer. Each fraction of eluate was examined by gel electrophoresis and the most purified and concentrated extracts were pooled. AGEs were prepared as previously described [21] by incubating 0.5 mM protein (BSA or HSA) with increasing concentrations of glucose (0, 5, and 25 mM) and in PBS, pH 7.4, under sterile conditions and nitrogen gas in capped vials at 37°C for 3 weeks or with methylglyoxal (10 mM) for 2 days. The proteins were dialyzed against PBS, pH 7.4, and sterile-filtered with 0.2-μm Millipore filters. Endotoxin content as assessed by an in vitro toxicology assay kit (E-TOXATE, Sigma) was below a detectable level (0.03 endotoxin unit/ml).

Purification of albumin from diabetic and nondiabetic patients

Purifications of albumin were conducted using pooled serum from three diabetic (% HbA_{1C} = 11.9 ± 1.2) and three nondiabetic (% HbA_{1C} = 4.83 ± 0.31) patients. Fasting blood glucose concentrations were comprised between 0.73 and 1 g/L for the group of nondiabetic persons, whereas it was higher (1.1 g/L) for diabetic patients. Isolation of serum albumin from human fresh plasma was performed as previously described and by using an affinity chromatography (Cibacron blue 3G; Amersham Cat. No. 17-0948 01).

Structural studies of AGEs: Measurements of fluorescence and absorbance

AGE-related modifications were determined by assessing the production of fluorescent compounds or glycophore [23] and by measuring the "browning color" resulting from the nonenzymatic glycosylation of albumin [24]. The formation of glycophore associated with the glycoxidation process in albumin was monitored at excitation and emission wavelengths of 355 and 410 nm, respectively, by using a microplate fluorometer FLUOstar OPTIMA (BMG Labtech, France). The brown color constitutes one of the qualitative properties of AGEs [25] and was analyzed by measuring the absorbance at 420 nm using a UV-visible spectrophotometer and expressed as absorbance per μg protein.

Oxidative modification of SH

Thiol groups in native or modified albumin were measured by Ellman's assay using 5,5′-dithiobis, 2-nitrobenzoic acid (DTNB) [26]. Briefly, 250 μl of albumin samples (in 0.1 M Tris-HCl, pH 8.0, EDTA 5 mM) was incubated with 3 vol of 0.5 mM DTNB. Considering the interference of browning state of modified albumin, the concentration of free thiol was calculated with a regression model by using the partial least-squares method (PLS) [27]. The PLS model was optimized by using 22 standard absorbance spectra recorded, between 250 and 500 nm, with a PowerWave microplate spectrophotometer (BioTek, USA). These standards correspond to various concentrations of L-cysteine (10 to 100 nmol) mixed with a browning solution at different concentrations. Results were expressed as the number of free –SH groups per mole of BSA.

REM study

Modifications of albumin (BSA or HSA) after glycoxidation were analyzed by native polyacrylamide gel (12% of acrylamide) and stained by Coomassie blue according to Laemmli's method [28]. Relative electrophoretic migration (REM) was determined for the different AGEs by calculating the migration ratio of the modified albumin monomer with the corresponding native albumin monomer (albumin which had not been previously incubated) [21].

Mass spectroscopic analyses

All mass spectroscopic measurements were performed at the Molecular Biophysics Unit (MBU) of the Indian Institute of Science (Bangalore, India). Molecular weights of both glycoxidized and nonglycoxidized samples were determined by electrospray ionization mass spectrometry (ESI/MS), while tryptic digestions were analyzed by MALDI-TOF-TOF.

ESI/MS was performed on Agilent 1100 LC coupled to Bruker Esquire3000plus operating in positive-ion mode. Complete ESI/MS settings were high voltage capillary, 4000 V; high voltage end plate offset, −500 V; nebulizer gas pressure, 28 psi; dry gas flow rate, 7.00 L/min; dry gas temperature, 340°C; capillary exit, 196.0 V; and Trap Drive, 120.6. MALDI/MS measurements were performed using UltraflexTOF/TOF from Bruker Daltomics (Germany). The setting condition of the mass spectrometer was as follows: reflection positive ion mode, accelerating voltage, 20 KeV. Ions were generated by the emission of nitrogen laser at a wavelength of 337 nm with 50 Hz repetition rate. Generally, an average of 100–150 laser shots is required to record a mass spectrum. Sinapinic acid (50 mg/ml in 50% acetonitrile, 9% trifluoroacetic acid [TFA], in water) was used as the matrix. The target plate used was the ground steel plate with 384 wells. Triplicate MALDI measurements were made for each sample for the estimation of reproducibility. Before enzymatic digestion with trypsin, AGEs samples were reduced with 100 mM DTT at 95°C for 5 min. After alkylation with a solution of 100 mM iodoacetamide for 20 min at room temperature, the samples were incubated with trypsin (200 μl of a 0.1 μg/μl solution) for 3 h at 37°C followed with an additional incubation overnight at 30°C. A total of 0.5 μl of digestion solutions was added to the same volume of matrix solution and the resulting mixtures were completely dried before acquisition.

Copper-binding test

The capacity of albumin for copper ion binding can be measured spectrophotometrically by the use of bathocuproinedisulfonic acid (BC, Fluka No. 11870) [29,30]. AGE preparations of BSA and HSA (0.2 μM in NaCl 0.15 M) were incubated in triplicate for 2 h with 10 μM $CuSO_4$. Then an overnight dialysis against NaCl 0.15 M was performed and a BC solution (1.2 mM) was added to each sample to a final concentration of 400 μM, followed by the addition of 1 mM sodium ascorbate solution. Incubation for 5 min at room temperature facilitated the complete reduction of Cu(II) to Cu(I) bound to albumin and resulted in the formation of BC–Cu(I) complex, which was read at 480 nm with a HP Model 8453 UV-visible spectrophotometer. The concentrations of copper bound to modified albumin were calculated using a calibration standard curve by increasing the concentrations of $CuSO_4$ up to 50 nM.

Hemolysis test of red blood cells

The antioxidant properties of AGEs were examined with the free radical-induced blood hemolysis test [31]. Human blood samples were obtained from the Biochemistry department of the local hospital center (CHD Felix Guyon) and were taken on EDTA substrate as anticoagulant. Then plasma was removed and erythrocytes were washed with an isotonic solution (NaCl 0.15 M). Each well of a 96-well plate was filled with 100 μl (about 1×10^8 erythrocytes, 400,000 cells/μl final concentration) of a diluted red blood cell solution (1/10 in 0.15 M NaCl). Different albumin samples (10 μM final concentration) were added in triplicates. Hemolysis was started by adding 0.45 M 2,2′-azobis(2-amidinopropane) (AAPH) in each well. Turbidimetry at 450 nm was recorded every 10 min using a 37°C thermostated microplate reader. Results were expressed as 50% of maximal hemolysis time (HT_{50} in min) which represents the total defense against free radicals in human and animal models submitted to oxidative stress [32].

Cell culture of THP1

Human monocytic leukemia cell line THP1 (ATCC TIB-202) was cultivated in RPMI 1640 medium with 10% SVF, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericine B (0.5 μg/ml). Cells were grown in a 5% CO_2 incubator at 37°C.

For all experiments, cells were plated in triplicate for each condition, at 10^4 cells/100 μl per well in sterile 96-well plates (for cytotoxicity analyses), at 2×10^5 cells/ ml per well in sterile 24-well plate (for ROS assays, Western blot, and proteasome activity measurement experiments). The culture plates were incubated for 24 h before adding different AGE preparations at a final concentration of 10 μM. Thereafter, cells were maintained in the humidified CO_2 incubator for 16 h before further analyses. Before proteasomal activity measurements and carbonyl Western blots, cells were washed thrice with PBS and were treated at 4°C for 30 min with lysis buffer (25 mM Tris-HCl, pH 8.3, 10 mM KCl, 1 mM DTT, 1 mM EDTA, 1% Triton X-100) without protease inhibitor. Cell lysates were then centrifugated and protein concentrations were determined in the supernatant by the BCA method [33].

Proliferation assay

The MTT assay, using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), was chosen to evaluate the effect of AGEs on THP1 cell proliferation [34]. This assay is based on the cleavage of yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which can be photometrically quantified. An increase in the number of living cells results in an increase in total metabolic activity, which leads to a higher color formation. After overnight cell incubation in the presence of different reagents, 20 μl of the MTT dye (5 mg/ml) was added into each well following by 4 h of incubation. After discarding of the media, 150 μl of isopropanol was added into each well and plates were agitated in the dark for 30 min to solubilize dark blue formazan crystals. The plates were read using a microplate reader at a wavelength of 595 nm. The negative control well (medium alone with no cell and no reagent) was used for zeroing absorbance. The control sample corresponds to THP1 cells incubated in the medium containing no albumin. The results were expressed as the percentage of viable cells with respect to the control.

Cytotoxicity: Lactate dehydrogenase (LDH) assay

To determine any possible toxic effect of AGEs on THP1 cells, the lactate dehydrogenase assay was performed [35]. This assay is based on the measurement of LDH released by dead cells in the culture medium. After overnight incubation of THP1 cells, media were collected and centrifuged at 3000*g* at 4°C for 5 min. The supernatants (medium) were collected in fresh tubes. A total of 120 μl of LDH assay lysis solution was added to the cells and tubes were put at 37°C for 45 min. Tubes were centrifuged at 250*g* for 4 min and supernatants (lysates) were transferred into a new tube. A total of 100 μl of supernatants (medium and lysates) was used for the measurement of LDH activity according to the manufacturer's instructions (Sigma No. TOX7). The reaction was performed in a new 96-well plate and absorbance at 492 and 690 nm (control) was read using the microplate reader.

Viable cell counts

ACScan flow cytometer (Becton-Dickinson) was used for the relative counting of viable THP1 cells. Equal amounts of R-phycoerythrin-labeled beads (CaliBRITE PE-beads, 5 μm; BD Biosciences) were added to each cell suspension just before the FACS analysis. An electronic gate was set to count the PE-labeled beads. All THP1 cells and PE bead events were recorded, and when the number of counted PE-labeled beads reached 2000, the counting process was stopped. A particular cell population was selected by gating and was identified by its typical location in a FSC (forward scatter) vs SSC (side scatter) graph. Results were expressed as the percentage of cells with respect to the control.

Measurements of proteasome activity

Chymotrypsin-like, trypsin-like, and caspase-like activities of proteasome were assayed using fluorogenic peptides (from Sigma) Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA at 25 μM), *N*-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-MCA at 40 μM) and *N*-Cbz-Leu-Leu-Glu-β-naphthylamide (LLE-NA at 150 μM), respectively [36]. Assays were carried out with approximately 50 μg of cell lysate in 25 mM Tris-HCl (pH 7.5) and the appropriate substrate at 37°C for 0–30 min incubation. The fluorescence of aminomethylcoumarin and β-naphthylamine products was determined at excitation/emission wavelengths of 350/440 and 333/410 nm, respectively, using a microplate spectrofluorometer reader (Fluostar, BMG France). Peptidase activities were measured in the absence or in the presence (20 μM) of the proteasome inhibitor MG132 (*N*-Cbz-Leu-Leu-leucinal) and the difference between the two values was attributed to proteasome activity.

Western blots

Carbonylated proteins were analyzed using the Oxyblot kit (Oxyblot Detection, Chemicon International Inc.) [37]. Briefly, about 5 μg of proteins (1 μg/μl) was denaturated by 5 μl 12% SDS for 10 min at room temperature. Samples were then treated with 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl for 15 min at room temperature

and then neutralized. The derivatized proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The primary antibody used was directed against the 2,4-dinitrophenol moiety and detection was performed using the ECL reagent (GE Healthcare). The monoclonal antibody 12G10 (anti-α-tubulin) was developed by Joseph Frankel and E. Marlo Nelsen. It was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, Iowa. The rabbit polyclonal antibody LMP2 was obtained from Santa Cruz (SC 28809). Rabbit anti-RAGE polyclonal antibodies were obtained from Sigma (R5278). Rabbit anti-CML polyclonal antibodies were obtained from abcam (ab27684).

Signal quantifications from images were determined using the freeware ImageJ (version 1.32j), available from the internet website: http://rsb.info.nih.gov/ij/.

Determination of ROS production

A 20 mM solution of H_2DCFDA was added to the cells at a final concentration of 10 μM, 30 min before the end of THP1 cell incubation with the different treatments. Then the cells were washed with RPMI medium and the fluorescence of the oxidized H_2DCFDA (DCF) was measured by FACS with fluorescence excitation at 488 nm (blue laser) and an emission at 530 nm (FL1). THP1 cells were gated for analysis based on light-scattering properties and DCF fluorescence intensity was assayed after counting 10,000 events. The results were expressed as the percentage of labeled cells with respect to the signal obtained for the positive control (THP1 cells incubated with PMA).

Statistical analysis

Data were expressed as the means ± standard deviation (SD) from a minimum of three experiments. Statistical significances were determined using one-way ANOVA (followed by the Tukey test). Two-way ANOVA was used for multiple comparisons, with P value less than 0.05 required for significance level.

Results

Characterization of AGE preparation

Serum albumin (HSA) was purified from fresh human plasma by following different steps of dialysis and affinity chromatography, and it was compared with commercial bovine serum albumin by native PAGE electrophoresis. As shown in Fig. 1a, electrophoresis profiles of commercial BSA and purified HSA gave striking differences. Contrary to the commercial BSA, albumin purified from human plasma exhibited only one single band relative to the monomeric form which was not accompanied by dimeric or oligomeric forms. AGEs were prepared by the treatment of these albumins with glucose (5 and 25 mM) and methylglyoxal (10 mM), and their absorbance was measured at 420 nm. Fig. 1b features the increasing level of fluorescence of BSA and HSA AGEs. This fluorescence is attributed to the formation of glycophore and is significantly higher for BSA_{MGO} (+1500%, $P<0.001$, vs BSA_{G0}) and HSA_{MGO} (+200%, $P<0.001$, vs HSA_{G0}). This increase in fluorescence was associated with a parallel rise of absorbance at 420 nm, which is attributed to browning reaction. Certainly, both pigmented and fluorescent products [24] were formed by nonenzymatic glycosylation. As seen in Fig. 1c, only the AGE solutions obtained by incubations with methylglyoxal gave significant enhancement in absorbance for BSA_{MGO} (+68%, $P<0.001$, vs BSA_{G0}) and for HSA_{MGO} (+21%, $P<0.05$, vs HSA_{G0}). To assess whether the degree of modification of HSA was pathophysiological, albumin from diabetic and nondiabetic patients had been isolated and used as the internal control. Similar increases in browning absorbance at 420 nm were noted for the albumin purified from diabetic patients or MGO-incubated HSA when compared with the respective controls (Fig. 1c)

To further characterize the oxidative modifications in our preparations, we examined whether the content of free thiol groups in albumin was altered by the glycoxidation process. The determination of free thiol groups was performed according to Ellman's dosage and results are shown in Fig. 1d. Regardless of the type of albumin, the results showed a significant decrease in the number of free thiol groups following the increasing concentration of glucose used for the incubations. In comparison with albumin incubated without glucose, this decrease was more obvious for BSA_{MGO} (−90%, $P<0.001$), but not for HSA_{MGO} (−14%). The decreased levels of reduced protein sulfhydryl groups were attributed to the oxidation of residue Cys34 and indicated the changes in protein conformation associated with the glycoxidation process [38]. Surprisingly, average values of 0.55 (0.41) free -SH per mole of BSA (HSA) were obtained for the untreated protein (no glucose) instead of one –SH group as expected for 1 mol of albumin [4]. In healthy adults, approximately 70% of Cys34 in albumin contains a free sulfhydryl group, and the rest forms a disulfide with several compounds like cysteine, homocysteine, or glutathione [39]. This suggests that commercially available BSA is already oxidized at residue Cys34, and the incubation of BSA or HSA in PBS, pH 7.4, at 37°C over a period of 3 weeks is sufficient to induce a decrease in the number of reduced thiol groups [21].

In a previous study [21], we showed that oxidation and glycation phenomena can affect the net charge of a protein. Different AGE preparations have been separated by native PAGE electrophoresis. Under these conditions, the protein charge would be the main factor affecting its migration. The native gel in Fig. 1e features the migration profiles for the different BSA and HSA preparations. Relative electrophoretic migration was determined by calculating the ratio of migrations of albumin monomer with nontreated albumin. Results are shown in Table 1. Significant increases in REM were observed for both albumins. A slight nonsignificant increase was noted for the HSA purified from diabetic patients in comparison with that purified from control subjects. With a slight increase for albumin incubated with glucose (+7.5% [+7%] for BSA_{G25} [HSA_{G25}], respectively, $P<0.05$ vs BSA_{G0} [HSA_{G0}]), a more pronounced rise was observed for BSA_{MGO} (+23%, $P<0.001$ vs BSA_{G0}) and HSA_{MGO} (+10.5%, $P<0.05$ vs HSA_{G0}). In further Western analysis, we studied the appearance of carbonyl groups (Fig. 1f) in glycoxidized albumin by using the principle of carbonyl assay developed by Levine et al. [37]. The Western blot revealed a noteworthy increase in protein carbonyl after modification with methylglyoxal in both treated albumins. Measurements of density indicated a significant increase in carbonyl content for HSA and BSA incubated with MGO. Besides, the level of carbonylation was higher for BSA AGEs than for HSA AGEs. Fig. 1g shows a higher carbonyl accumulation in the albumin purified from diabetic patients when compared with protein from control subjects.

Among the different AGEs, CML and CEL constitute the most common epitope. From Fig. 1h, it can be seen that Western blot using antibodies directed against CML showed an enhanced signal for this AGE modification for albumin incubated with increasing concentrations of glucose and for MGO-treated albumin. Several studies have shown that methyglyoxal can react with albumin to form AGE structures such as N^{ε}-(carboxyethyl)lysine (CEL) while glucose gives rise to N^{ε}-(carboxymethyl)lysine (CML) [40,41]. The very high signal observed for BSA_{MGO} or HSA_{MGO} suggests that polyclonal anti-CML antibody used in this study recognized not only CML but also cross-reacted with CEL, as already shown in another previously published study [42].

Mass spectroscopy analyses

Modifications of different albumin preparations, including native BSA and HSA, were assessed by ESI/MS, which indicated the number of

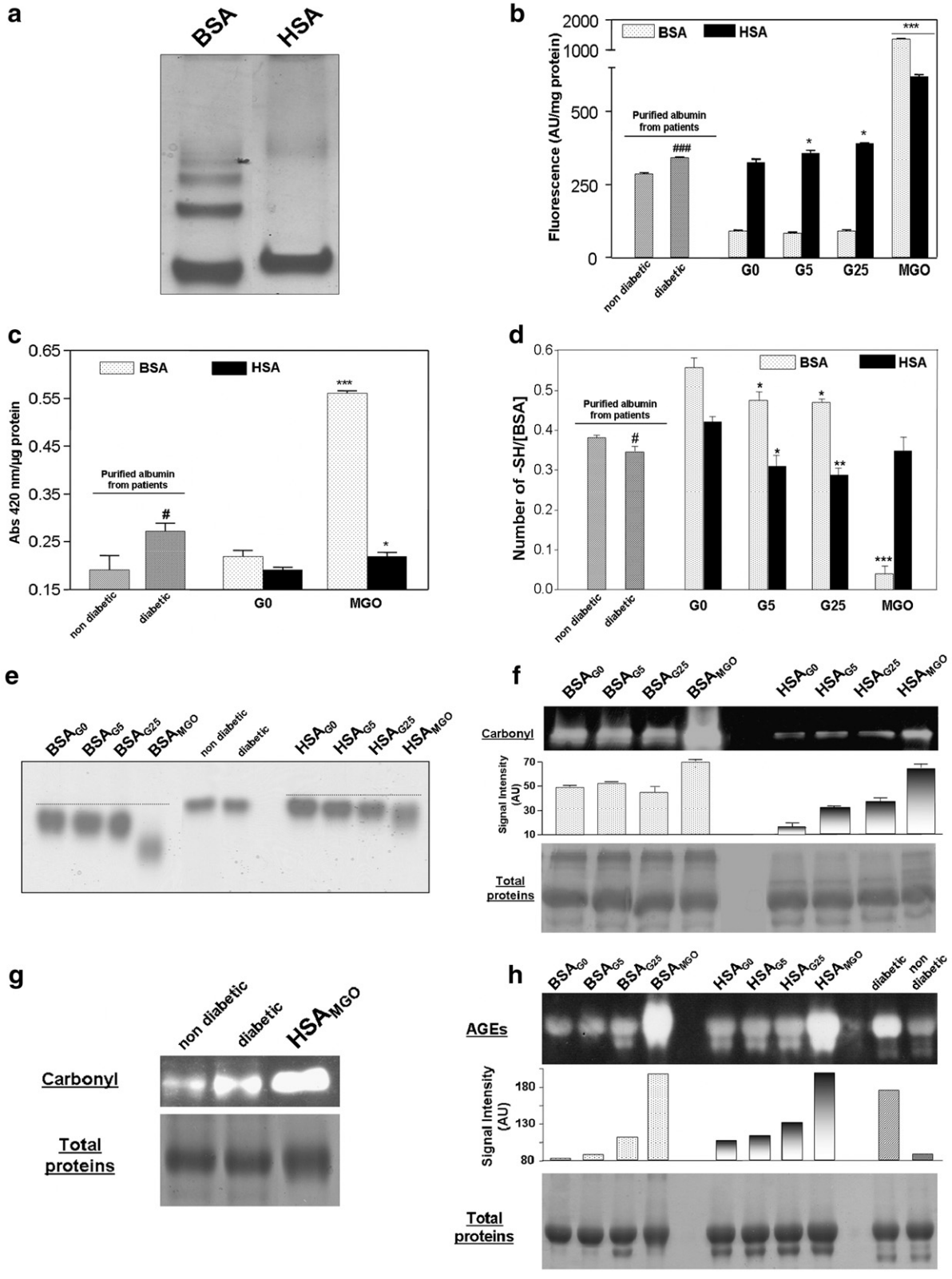

Fig. 1. Characterization of BSA and HSA AGEs. (a) Electrophoretic migration profile of commercial BSA and purified HSA (PAGE 12%). (b) Fluorescence of AGEs at 355/410 nm expressed in arbitrary units of fluorescence per mg protein. (c) Absorbance at 420 nm for albumin incubated without glucose (G0) or with 10 mM methylglyoxal (MGO) and for albumin purified from diabetic and nondiabetic patients. (d) Thiol group contents in the different preparations of albumin evaluated with Ellman's method. (e) The Coomassie staining of the analysis of BSA and HSA preparations by native PAGE electrophoresis (12%). (f) Oxidative modification of glycoxidized albumin as assessed by the carbonyl assay with signal intensity (AU) of carbonyl spots. (g) Oxidative modification of albumin purified from diabetic and nondiabetic patients as assessed by the carbonyl assay. (h) AGE content in albumin preparations as assessed by Western blotting with the corresponding signal intensities (arbitrary unit). All data in (b) to (d) are expressed as mean ± SD ($n=4$) and statistical analyses were performed using one-way ANOVA for multiple comparisons. * Effect of the glycoxidation of BSA [HSA] (vs BSA_{G0} [HSA_{G0}]): $^{***}P<0.001$, $^{*}P<0.05$. $^{\#}$Effect of diabetic pathology (vs non diabetic): $^{\#\#\#}P<0.001$, $^{\#}P<0.05$.

Table 1
Relative electrophoretic migration of glycoxidized bovine and human serum albumin

	BSA_{GO}	BSA_{G5}	BSA_{G25}	BSA_{MGO}
REM	1.068 ± 0.008	1.122 ± 0.009*	1.149 ± 0.008***	1.317 ± 0.036***
	HSA_{GO}	HSA_{G5}	HSA_{G25}	HSA_{MGO}
REM	1.015 ± 0.011	1.065 ± 0.005	1.086 ± 0.005*	1.120 ± 0.016*

Relative electrophoretic migration (REM) of the different preparations of BSA and HSA was evaluated as described under Materials and methods. Data of REM are represented as mean ± SD ($n = 3$) and statistical analyses were performed using one-way ANOVA. * Effect of the glycoxidation of BSA [HSA] (vs BSA_{G0} [HSA_{G0}]): ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

glucose molecules or methylglyoxal condensed on the protein (Table 2). The average molecular mass specified in the Table corresponds to the mean of different masses of proteins exhibiting different degrees of modification. The most abundant components are also featured in Table 2. As expected, nonenzymatic glycosylation of albumin with glucose or methyglyoxal induced an increase in its average molecular weight, with variable extent, depending on the nature of glycoxidation. Molecular masses of native BSA and HSA were approximately 66438 and 66567 Da, respectively. The most abundant species in BSA_{G5} and HSA_{G5} samples were the nonmodified (native) albumin (no mass variation). The molecular mass of the most abundant species in HSA_{G25} was increased to approximately 167 Da corresponding to the protein condensed with one glucose unit. Definitely, the condensation of one glucose or methylglyoxal molecule to the protein leads to a mass increase of 162 or 54 Da, respectively. Similarly, the molecular mass of predominant species for HSA_{MGO} was increased to 166 Da, which probably corresponds to three methylglyoxal units attached to the protein.

Different HSA and BSA samples were also digested by trypsin. MALDI spectra for the trypsic proteolyses of native, glycoxidized (with methylglyoxal), and diabetic HSA are shown in Fig. 2. The comparison of nonglycoxidized (HSA_{G0}) and glycoxidized (HSA_{MGO} and HSA_{diab}) albumin clearly revealed several differences: first, the main obvious change was the disappearance of most abundant ionic species (for nonglycoxidized albumin digest) at *m/z* 1623.5, and second, the appearance of several predominant ionic species (for glycoxidized albumin digest) at *m/z* 673.6, 875.4, 1149.5, and 1546.6. Some other peptides of HSA_{G0} digests still remained at low abundance in glycoxidized digests (HSA_{MGO} and HSA_{diab}), particularly at *m/z* 927.5, 1342.5, and 1931.5. In conclusion, even if the tryptic patterns were not exactly the same, an important similarity was evidenced between in vitro-modified albumin (HSA_{G25} and HSA_{MGO}) and in vivo diabetic HSA digest profiles.

In summary, the main modifications in BSA and HSA in terms of oxidative and structural parameters were observed in the presence of albumin incubated with methylglyoxal. Besides, a comparison between in vitro-modified and in vivo-modified albumin (diabetic) showed similar changes in the protein structural properties. This was more significant when structural changes were compared between HSA_{diab} and HSA_{G25}.

In the following experiments, antioxidant properties and effects of glycoxidized albumin on THP1 cells were investigated.

Impairment of antioxidant properties of BSA and HSA after glycoxidation

To examine whether the glycoxidation treatment had any effect on the antioxidant properties of albumin, different albumin preparations previously characterized were submitted to the free radical-induced hemolysis test and their copper-binding capacity was evaluated. The antioxidant properties of albumin samples were investigated using the red blood hemolysis test either in the absence

Table 2
ESI mass analysis of in vitro-modified BSA and HSA

	Major Component	Molecular Mass	Abundance (%)	Mass Increase (Da)		Component	Molecular Mass	Abundance (%)	Mass Increase (Da)
BSA_{G0}					**HSA_{G0}**				
	A	**66438.47**	**100**			A	**66567,43**	**100**	
	B	**66552.06**	**44.52**	+113.6		B	**66746,18**	**59,01**	+ 178,7
Average Molecular Mass (Da)		66524			Average Molecular Mass (Da)		66613		
BSA_{G5}					**HSA_{G5}**				
	A	**66444.2**	**100**	+ 5,7		A	**66567,18**	**100**	- 0,2
	B	**66596.9**	**34.97**	+ 158,2 (**+ 1 glc**)		B	**66653,06**	**60,95**	+ 85,6
						C	**66730,66**	**56,06**	163,2 (**+1 glc**)
Average Molecular Mass (Da)		66587	→	+ 63	Average Molecular Mass (Da)		66795	→	+ 182
BSA_{G25}					**HSA_{G25}**				
	A	**66518.92**	**100**	+ 80.45		A	**66734,78**	**100**	+ 167.3 (**+1 glc**)
	B	**66601.67**	**74.26**	+ 163.2 (**+1 glc**)		B	**66900,59**	**79,96**	+ 333.1 (**+2 glc**)
	C	**67091.31**	**65.8**	+ 652.8 (**+4 glc**)		C	**66564,44**	**73,97**	- 2.9
Average Molecular Mass (Da)		66714	→	+ 190	Average Molecular Mass (Da)		66925	→	+ 312
BSA_{MGO}					**HSA_{MGO}**				
	A	**66681.65**	**100**	+ 243.1		A	**66733,55**	**100**	+ 166.1 (**+3 mgo**)
	B	**66604.63**	**81**	+ 166.1 (**+3 mgo**)		B	**66570**	**91,97**	+ 2.5
	C	**66760.12**	**63.35**	+ 321.6 (**+6 mgo**)		C	**66625,72**	**93,18**	+ 58.2 (**+1 mgo**)
	D	**66444.84**	**66.06**	+ 6.3		D	**66885,87**	**85**	+ 318.4 (**+6 mgo**)
Average Molecular Mass (Da)		67713	→	+ 1189	Average Molecular Mass (Da)		66868	→	+ 255

Average molecular mass (Da) of the different preparations of BSA and HSA was obtained by ESI/MS. Abundance of the different components was determined (%). Mass increase was calculated by comparison with native BSA (66438 Da) and HSA (66567 Da). Number of glucose (glc) or methylglyoxal (mgo) units attached to the protein was specified.

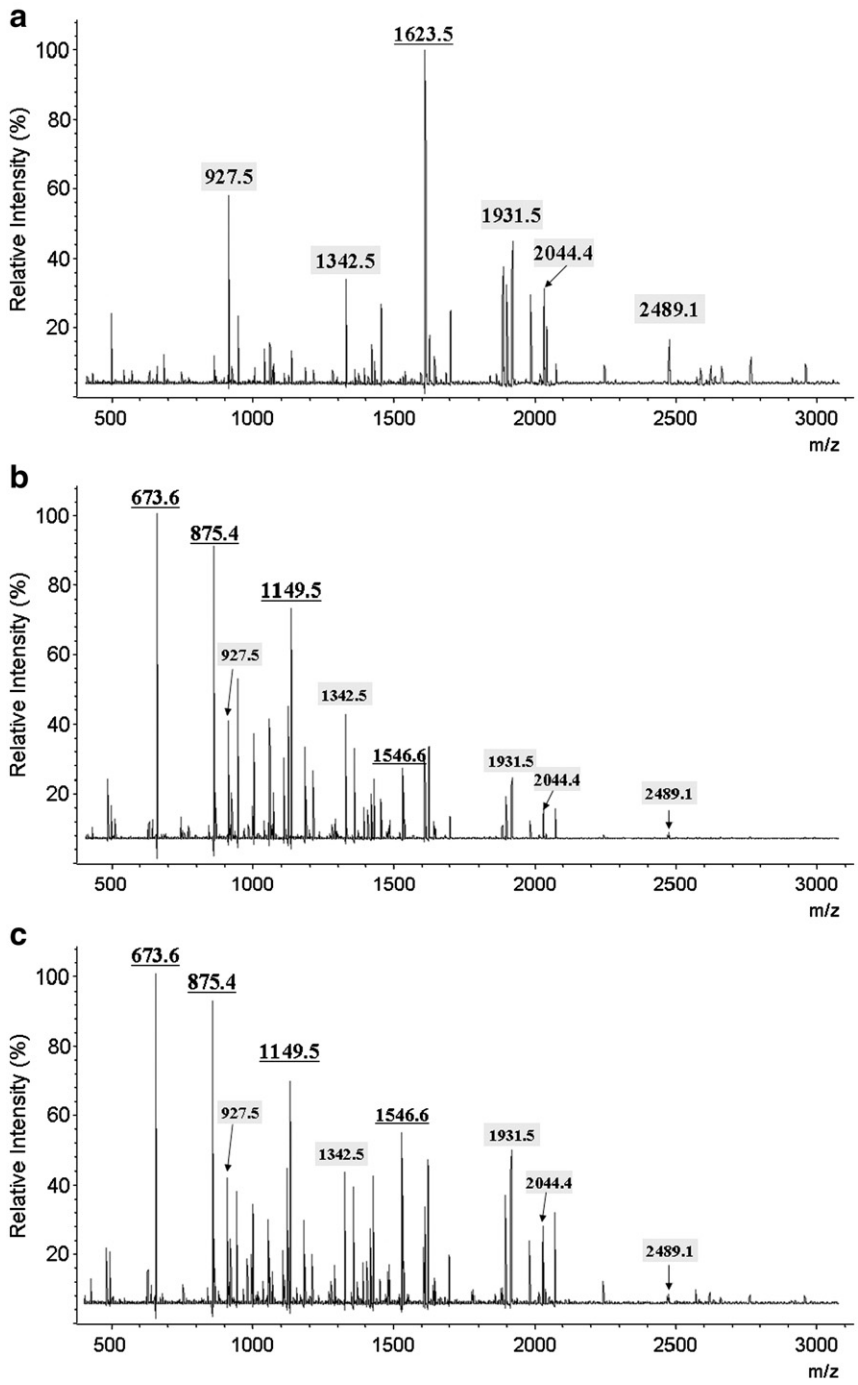

Fig. 2. Comparisons between peptidic profiles of glycoxidized and nonglycoxidized HSA. MALDI spectra of tryptic digest of (a) HSA_{G0}, (b) HSA_{MGO}, and (c) HSA of diabetic patients. The ionics species with underlined *m/z* correspond to peptides present in the three samples.

or in the presence of 10 μM protein. Typical hemolysis curves obtained for control (PBS), HSA_{G0}, and HSA_{MGO} are illustrated in the insert of Fig. 3a. As expected, the antioxidant property of nonmodified albumin was evidenced by delayed hemolysis in comparison with control, and this was observed either with BSA_{GO} (curves not shown) or with HSA_{GO}. The free radical scavenging properties of different albumin preparations, symbolized by hemolysis half-times (HT_{50}), are shown in Fig. 3a. On the one hand, the protective effect observed for BSA_{G0} (+16%, $P<0.001$ vs PBS) and for HSA_{G0} (+26%, $P<0.001$ vs PBS) was progressively lost after the glycoxidation of protein (Fig. 3a). On the other hand, a collapse in the antioxidant property of the protein was noted for both BSA_{MGO} and HSA_{MGO}. In comparison with HSA_{G0}, albumin modification by methylglyoxal induced a 24% reduction ($P<0.001$) in the antioxidant activity of protein. A similar reduction was observed for modified BSA with 26% ($P<0.001$) reduction in the antioxidant activity. Remarkably, an increase in antioxidative properties was observed for in vivo diabetic HSA compared with nondiabetic albumin.

The antioxidant activity of albumin was also related to its capacity to bind metal such as copper ions. By using bathocuproinedisulfonic acid to assay bound Cu(II), we determined copper-binding capacities (nM/μmol) of modified albumin (Fig. 3b). Surprisingly, the results indicated a progressive rise in copper-binding capacity of BSA with glycoxidation process. This rise reached 260% for BSA_{MGO} ($P<0.001$, vs

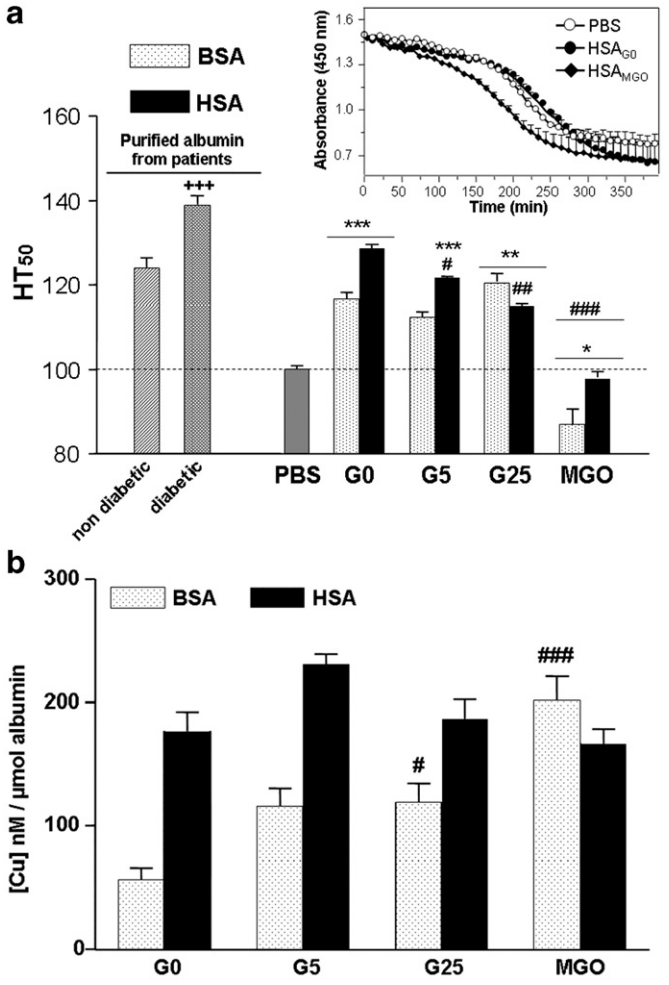

Fig. 3. Effects of glycoxidation on the antioxidant activities of albumin. (a) Impairment in the antioxidant activities of BSA and HSA as assessed by the free radical-mediated blood hemolysis test. Insert is an illustration of hemolysis curves obtained in the absence (○, control no HSA) or the presence of 10 µM HSA incubated with (♦) or without (●) methylglyoxal. Histograms represent half-time hemolysis (HT_{50} in min) expressed as means ± SD of three independent experiments. The dotted line facilitates comparison with control (no albumin). Statistical analyses were performed using one-way ANOVA for multiple comparisons. * Effect of BSA or HSA (vs control): $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. # Effect of glycoxidation (vs BSA_{G0} or HSA_{G0}): $^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$. + Effect of diabetic pathology (vs non diabetic): $^{+++}P<0.001$. (b) Effect of glycoxidation on copper-binding capacities of BSA or HSA. Histograms represent copper-binding capacities of modified albumin (nM/µmol albumin). Data are expressed as mean ± SD ($n=3$) and statistical analyses were performed using one-way ANOVA for multiple comparisons. *Effect of glycoxidation (vs BSA_{G0} (HSA_{G0})): $^{\#\#\#}P<0.001$; $^{\#}P<0.05$.

BSA_{GO}), while it was less for BSA_{G25} (112%, $P<0.05$ vs BSA_{G0}). Argirova and Ortwerth, in their study on glycoxidized BSA, showed that highly glycoxidized (500 mM glucose) albumin was less capable of competing for copper ions in the presence of other ligands, suggesting that glycoxidized proteins might have diminished stability constants of their copper chelates [29]. For HSA, no significant variations in the copper-binding capacities of protein were observed after its modification by glycoxidation with an exception for $HSA_{G5,}$ where a slight increase was noted.

Effects of AGEs on THP1 cells

Before further analysis on the impact of AGEs on monocytes physiology, the effects of different AGE preparations on the viability of THP1 cells were assessed. The cytotoxicity effects were assayed by measuring cell proliferation and viability. In the subsequent experiments, THP1 cells were incubated with mild concentrations of AGEs in the presence of 10 µM native or glycoxidized albumin. First, the effect of characterized AGEs on the metabolic activity of THP1 cells was assessed in vitro by the MTT assay. The incubation with 10 µM albumin AGEs resulted in an increase in the viability of cells compared to PBS control as shown in Fig. 4a. It increased up to 22% ($P<0.01$) for BSA_{G25} and 20% ($P<0.05$) for HSA_{MGO} compared with control. Regardless of the nature of albumin preparations incubated in the presence of cells, no significant impairment in their viability was noted.

In parallel, the membrane integrity of THP1 cells submitted to AGEs was monitored in terms of cytoplasmic LDH activity of the

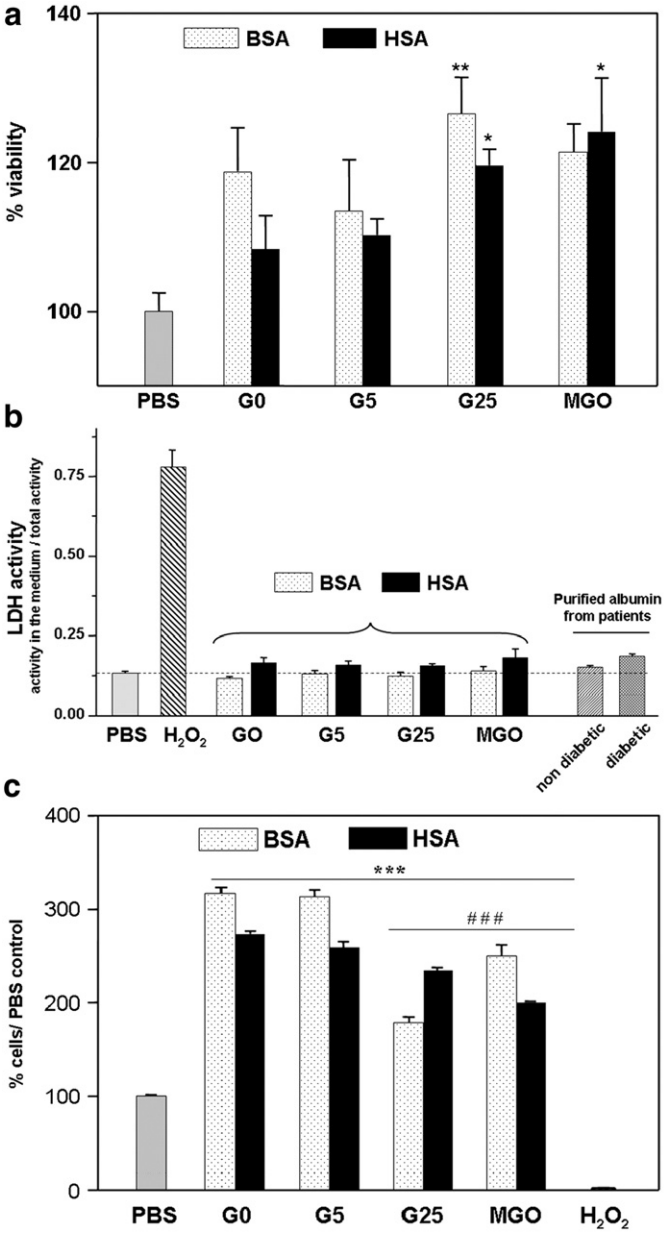

Fig. 4. Effects of AGEs on THP1 cells integrity. The relative viability (%) of THP1 cells incubated 16 h with (a) 10 µM AGEs and determined by MTT assay. (b) Effect of 10 µM AGEs on the release of lactate dehydrogenase in THP1 cells supernatant medium. Results are expressed as the percentage of LDH released in the medium relative to the total LDH activity present (medium plus cell lysates). (c) Viability of THP1 cells treated with 10 µM AGEs, expressed as the percentage of cells compared with PBS control and determined by a relative counting using a FACScan flow cytometer. In all experiments, the viability was compared with control (cells incubated with PBS) and positive control corresponds to cells incubated with hydrogen peroxide (1.5%). Data are expressed as mean ± SD ($n=3$) and statistical analyses were performed using one-way ANOVA for multiple comparisons. *Effect of BSA or HSA (vs control): $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. # Effect of glycoxidation (vs BSA_{G0} or HSA_{G0}): $^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$.

medium. The results of LDH activity are shown in Fig. 4b and no significant differences in LDH activity under each condition of cell incubation are observed. Contrarily to the viability results obtained with the MTT method, the direct cell count by FACS showed a decrease in the number of THP1 cells when incubated in the presence of albumin glycoxidized by 25 mM glucose or 10 mM methylglyoxal. On the one hand, as depicted in Fig. 4c, the decrease was approximately 70% [20%] ($P<0.001$ [0.001] vs BSA_{G0} [HSA_{G0}] for BSA_{G25} [HSA_{G25}] and about 27% [36%] ($P<0.001$ [0.001] vs BSA_{G0} [HSA_{G0}]) for BSA_{MGO} [HSA_{MGO}]. On the other hand, significant increase of viability was observed for cells incubated in the presence of either glycoxidized or nonglycoxidized albumin (BSA and HSA).

Effect on proteasomal proteolytic activities

The proteasome represents the major constituent of the proteolytic pathway for the degradation of oxidized proteins and it includes a minimum of three distinct peptidase activities: chymotrypsin-like, trypsin-like, and caspase-like activities [43].

Fig. 5a reports proteasomal activities measured in THP1 cells, which had been incubated in the absence (PBS) or the presence of 10 μM native or glycoxidized BSA. Significant increases in chymotrypsin-like activities (LLVY) were observed in glycoxidized-incubated cells. No significant variation in the trypsin-like activities (LSTR) was noted when the cells were incubated with native or glycoxidized bovine serum albumin. Regarding the caspase-like activities (LLE), a slight but significant reduction was observed when the cells were incubated in the presence of native BSA (BSA_{G0}) than in the absence of albumin (PBS). LLE activities were higher in glycoxidized BSA-incubated cells than in cells treated with native albumin (BSA_{G0}).

Effects of native or glycoxidized HSA on the proteasomal activities of THP1 (Fig. 5b) were quite different from those previously observed with glycoxidized and nontreated BSA. Significant increases in chymotrypsin-like activities were observed when the cells had been incubated with native or glycoxidized HSA than in the absence of the protein (PBS). Concerning the trypsin-like activity of the proteasome and in contrast to what was previously observed with BSA preparation-incubated cells, a significant reduction was evidenced in cells incubated with HSA_{G25} ($P<0.05$ vs HSA_{G0}). Caspase-like activity (LLE) was strongly impaired when cells were incubated with HSA_{MGO} (−30%, $P<0.001$ compared with HSA_{GO}). The different effects of HSA and BSA on proteasomal activity might be due to structural differences between BSA and HSA.

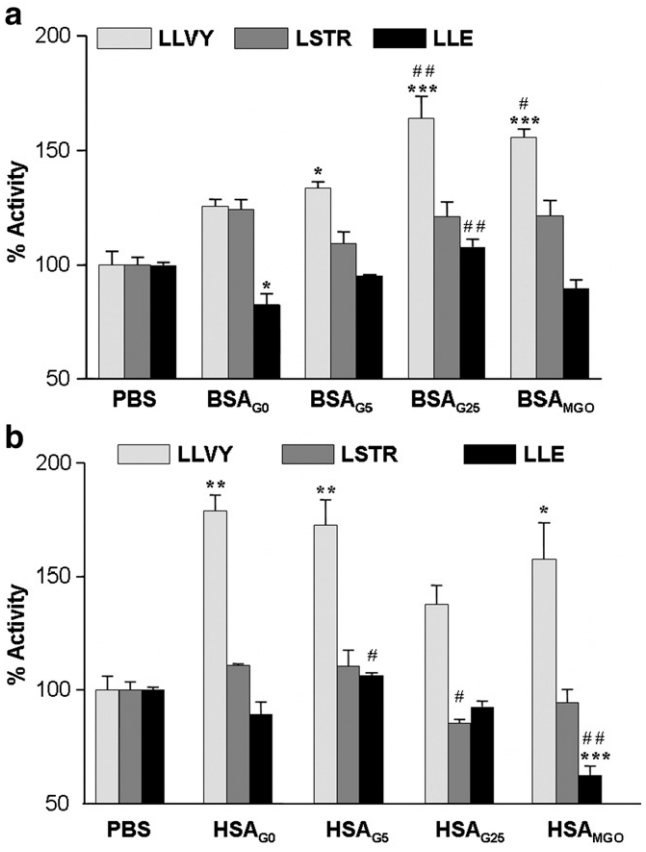

Fig. 5. Effect of AGEs on proteasomal activities. THP1 cells were incubated in the presence or absence (PBS) of 10 μM AGEs generated either from BSA (a) or HSA (b) for 16 h. Cells were washed and lysates were prepared as described under Materials and Methods. The caspase-like, chymotrypsin-like, and trypsin-like activities of the proteasome were assayed using the fluorogenic peptides substrates LLE-NA, LLVY-AMC, and LSTR-AMC, respectively, at 12.5 μM final concentration. Assays were carried out with 50 μg of cell lysates in 25 mM Tris-HCl (pH 7.5) and with the appropriate substrate at 37°C for 0–30 min incubation. The fluorescence of each sample was evaluated using a multiplate spectrofluorimeter (BMG Labtech) at excitation/emission wavelengths of 350/440 and 333/410 for aminomethylcoumarin and β-naphthylamine products, respectively. Peptidase activities were measured in the absence or the presence (10 μM) of the proteasome inhibitor MG132, and the difference between the two values was attributed to proteasome activity. Bars represent mean ± SD ($n=3$ to 8), P values were obtained using one-way ANOVA. *Effect of BSA or HSA (vs control): $^{***}P<0.001$, $^{*}P<0.05$. $^{\#}$ Effect of glycoxidation (vs BSA_{G0} or HSA_{G0}): $^{\#\#}P<0.01$, $^{\#}P<0.05$.

Effect of glycoxidized BSA and HSA on carbonyl accumulation and the expressions of RAGE and LMP2 by THP1

The investigation whether glycoxidized albumin might lead to an accumulation of carbonylated proteins in the cells was highly important. Carbonyl assay was performed on cells, which had been incubated for 16 h in the absence or the presence of 10 μM of different albumin preparations (Fig. 6). A protective effect of native albumin was observed, as the profiles for the carbonyl proteins were less intense when the cells were incubated with native albumin (BSA_{G0} or HSA_{GO}) compared to PBS control. As asserted by density measurements, an increase in the signal for carbonylated cellular proteins was noted in those cells previously incubated in the presence of the following glycoxidized albumins: BSA_{G25}, BSA_{MGO}, and HSA_{MGO}.

LMP2 constitutes a component of the eukaryotic multicatalytic proteinase complex, the proteasome. As the glycoxidized albumin-induced changes in proteolytic activities could be related to variations in the proteasome levels, Western blots were performed using antibodies directed against the LMP2 subunit of the enzymatic complex (Fig. 6). The proteasome expression was similar when cells were incubated with native or glycoxidized albumin. It is noteworthy that a slight increase and little decrease in LMP2 were observed when cells were incubated with BSA_{MGO} and HSA_{MGO}, respectively. This reduction in LMP2 expression in the cells treated with HSA_{MGO} might explain the significant alteration in the caspase-like activity of the proteasome in THP1 incubated with this modified albumin.

Concerning RAGE receptor expression, antibody used for the Western blot detected two bands in the 45 kDa range representing the RAGE protein and a ~25 kDa protein that was reported to be a nonspecific reaction. A stronger signal was observed in glycoxidized BSA than in native BSA or in PBS control. No significant variation in RAGE expression was evidenced in the cells incubated in the presence of the different preparations of HSA. Nevertheless a higher RAGE expression was noted in cells incubated with HSA_{GO} or HSA_{MGO}.

Reactive oxygen species

The oxidative stress in THP1 after 16 h incubation in the absence or presence (10 μM) of various albumin preparations was examined by using the dichlorofluorescein diacetate (DCF-DA) reagent [44]. After internalization by the cell, hydrolysis (removal of acetate moiety), and

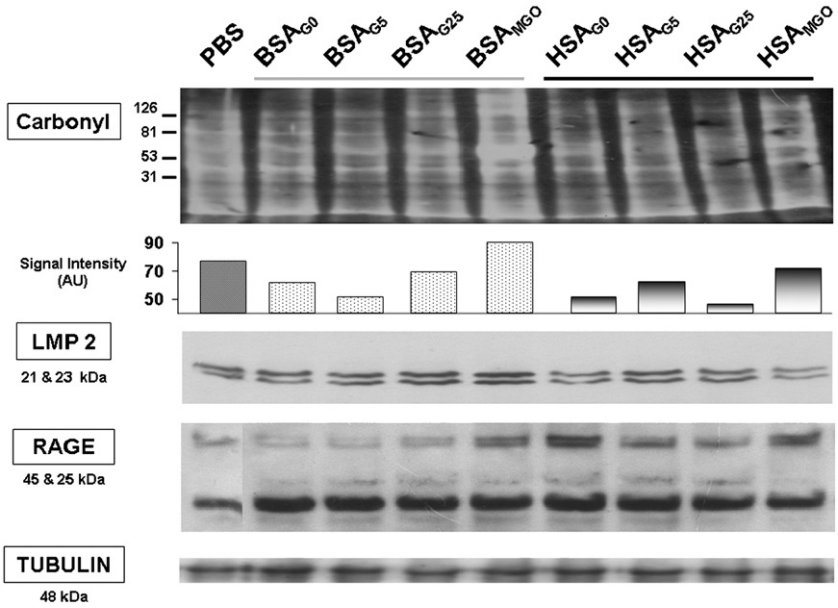

Fig. 6. Expression of LMP2, CD 36, and RAGE receptor by Western blot. THP1 cells were incubated for 16 h in the absence or presence of 10 μM native or glycoxidized albumin. Approximately 25 μg of total protein from lysates of treated cells was separated by SDS-PAGE and transferred to nitrocellulose membranes. Antibodies (see Materials and Methods) were used at a dilution of 1:1000 and visualized using the appropriate HRP-conjugated secondary antibody and the ECL system (GE Healthcare). Signal quantification of carbonyl was determined by using the freeware ImageJ.

oxidation by various oxidants, the nonfluorescent fluorescein derivatives (dichlorofluorescein, DCFH) will become DCF and emit fluorescence. As THP1 monocytes constitute nonadherent cells, DCF fluorescence was determined after cellular treatments by using a FACS technique. The cellular counts of fluorescence in Figs. 7a and 7b reveal a higher ROS formation in THP1 treated with MGO-modified albumin compared with respective controls (BSA_{G0} or HSA_{GO}). Results of ROS formation in THP1 incubated with the different albumin preparations are shown in Fig. 7c. Protective effects of native albumin are again evidenced here. A decreased fluorescence signal was observed when the cells were incubated with native albumin (BSA_{G0} or HSA_{GO}), compared to PBS control. An increase in the ROS formation was observed in those cells previously incubated in the presence of glycoxidized albumin and especially with BSA_{MGO} or HSA_{MGO}.

The results of statistical analysis using two-way analysis of variance (ANOVA) are summarized in Table 3. The two parameters analyzed in this test were albumin origin (BSA or HSA) and glycoxidation treatment (G0, G5, G25, and MGO). It was observed that the nature of albumin chosen (BSA or HSA) in the different experiments significantly affected the results at a structural or physiological level. Glycoxidation treatment significantly affected the results of the structural studies, antioxidant capacity of albumin, caspase-like activity of the proteasome, and ROS production in THP1 cells. Most interestingly, we addressed the question of interactions between the two parameters: "Do both parameters exert any interactions?" In most instances, for the majority of studies conducted, the answer to this question is "yes" as interactions between the two parameters were ascertained by the calculated *P* values inferior to 0.05.

Discussion

Elevated concentrations of circulating glycoxidized albumin demonstrate a typical feature of blood in diabetic patients [9]. Glycoxidized proteins alter tissue function and disturb cellular metabolism. Interaction of AGEs with several cell types has been shown to induce intracellular stress and leads to an increased production of cytokines or nitric oxide (NO), activate NFκB or heme oxygenase, produce lipid peroxidation products, and crosslink proteins [10,45]. Extensive studies have focused on the impact of AGEs on cell physiopathology. Nonetheless, only a few studies have reported the comparative effects of glycoxidation phenomenon on the structure of BSA and HSA and on the induced oxidative stress in cells. In the present study, we compared (i) the consequences of glycoxidation induced by patho- (25 mM) or physiological (5 mM) concentrations of glucose or methylglyoxal on the structure and antioxidative properties of BSA or HSA, (ii) the cellular impact of glycoxidized albumin on THP1 by the determination of cell viability, accumulation of carbonylated proteins, expression of RAGE, and the generation of oxidative stress in the cell.

Impact of glycoxidation on the structure of albumin

We previously reported some structural changes in albumin induced by the glycoxidation of protein. But, in these studies, BSA was incubated with high concentrations of glucose (100 mM), or in the presence of copper, or submitted to elevated temperature. Also, cells were incubated in the presence of high glycoxidized BSA concentrations (0.37 mM) [20–22,46]. Here, we tried to mimic either physiological or pathophysiological conditions by incubating HSA or BSA with glucose and methylglyoxal and by treating monocytes with modified albumin. Extensive care was taken for the incubation of albumin with sugar for 3 weeks, which corresponds to the half-time of the protein in the plasma. The major structural change in oxidized HSA from healthy human plasma occurred at the thiol group of Cys34 of reduced HSA [47]. Under our experimental conditions, purified HSA was less oxidized than commercial BSA (Fig. 1a), which is in accordance with another report detailing such structural differences in albumin preparations [38]. Structural information indicated the formation of a molten globule-like state of HSA after 21 days of incubation with 35 mM glucose [48]. Here, significant oxidations of thiol in BSA and HSA were induced, when the proteins were glycoxidized by 25 mM glucose. Cys34 could account for a large percentage (>55%) of oxidized thiol in human serum albumin obtained either from critically ill patients or from diverse commercial preparations [38]. Similarly, as assessed by the carbonyl assay, an increase in the carbonylation of the proteins was observed (Fig. 1f) and this is consistent with the previously reported results, where a significant increase of carbonyl groups in HSA was observed on

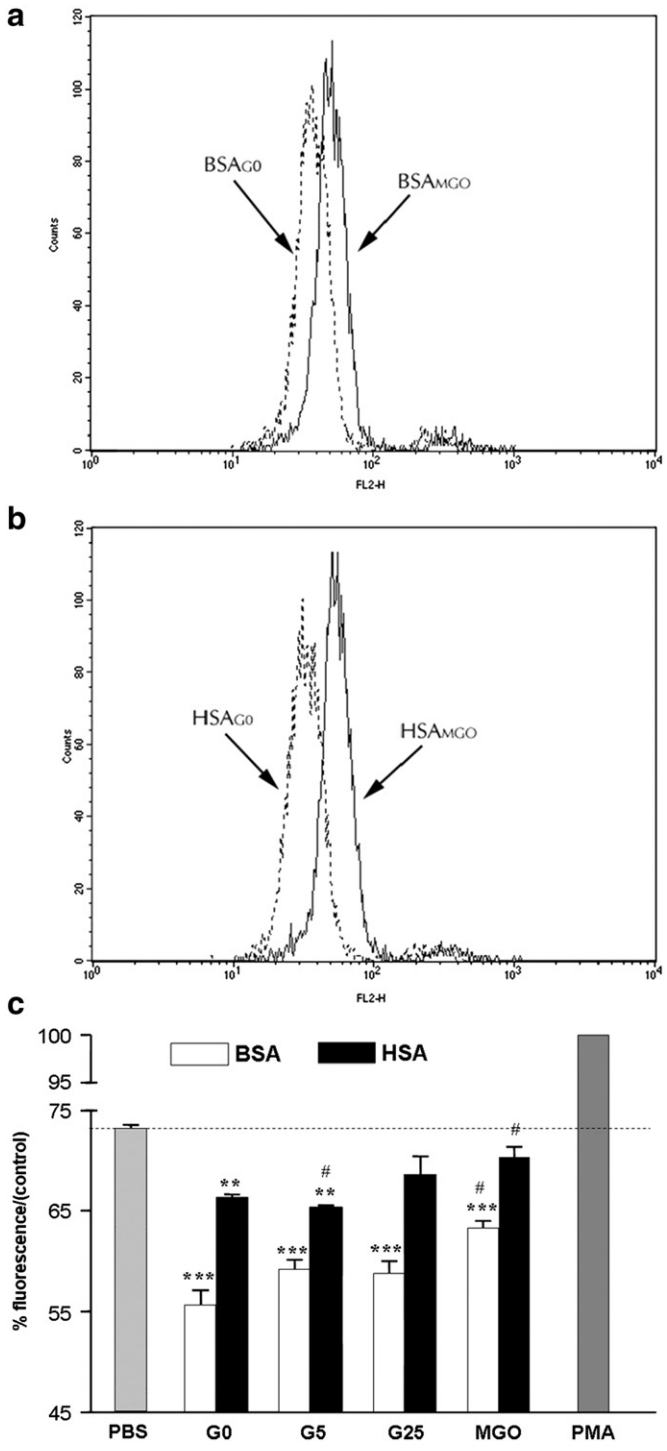

Fig. 7. Production of ROS in THP1 cells with AGEs. FACS analysis of DCF fluorescence of THP1 cells incubated overnight with (a) BSA and (b) HSA AGEs. The ratio of fluorescence levels of cells treated with albumin with methylglyoxal was compared with those without methylglyoxal or glucose. (c) Relative DCF mean fluorescence intensity for cells incubated with BSA and HSA AGEs. All fluorescence intensities are compared with positive control (cells incubated with 1 pg/ml PMA). Data are expressed as mean ± SD (n=3) and statistical analyses were performed using one-way ANOVA for multiple comparisons. *Effect of BSA or HSA (vs PBS control): ***P<0.001. # Effect of glycoxidation (vs BSA_{G0} or HSA_{G0}): # #P<0.01, #P<0.05.

glycoxidation for 20 days with 50 mM glucose [49]. Among carbonyl modifications of plasmatic proteins, methylglyoxal (MGO), a very reactive dicarbonyl compound, is increased in diabetes. Recent data suggest that MGO can strongly impair the structure and antioxidants properties of albumin in vitro, leading to a modified protein similar to that isolated from diabetic patients [50]. Here, utmost increases in glycophore fluorescence, protein carbonylation, and REM of albumin were observed in the MGO-modified protein.

Very recently, a diabetic patient with poor glycemic control was found to exhibit more than 94% of his albumin presenting a glycative state [51]. Our group recently showed that the incubation of BSA with 25 mM glucose for 7 weeks led to 59% of glycoxidized albumin [21]. Under the experimental conditions of this study, the protein structure of BSA was more drastically affected by glycoxidation than that of HSA.

Different impacts of glycoxidation on the antioxidant properties of BSA or HSA

Albumin, the most abundant protein in the plasma, could act as an important circulating antioxidant [7,52], in addition to several properties such as the transport of fatty acids and regulation of oncotic pressure [4]. Some previous studies showed that the integrity of albumin was necessary for the protein to exert efficient antioxidant activity [20,30,46]. For the first time, the effects of sole glycoxidation of BSA and HSA on the antioxidant activity of protein were assessed by the determination of their copper-binding and free radical-trapping capacities. Our present data are consistent with the previous observations made in 1999, which showed that the sole glycoxidation of BSA with increasing concentration of glucose (up to 500 mM glucose) had no effect on the free radical-trapping capacities of albumin, as assessed by the AAPH-induced test [20]. Impaired BSA antioxidant activities were observed only in copper-induced oxidized albumin previously glycoxidized [20]. A very significant alteration in the free radical-trapping capacity of BSA was observed (Fig. 3a) when the protein was modified by MGO. Faure et al. recently showed that antioxidant capacity of BSA determined by thiol measurement as well as in a cellular system (HeLa cells stressed by H_2O_2) was significantly altered after modification by MGO [50]. In human albumin, its sole glycoxidation with 25 mM glucose was sufficient to significantly impair the free radical-trapping capacity of protein (Fig. 3a). In addition, HSA modified by MGO did not exert any antioxidant activity. This impairment in the free radical-trapping capacity of HSA after glycoxidation was not associated, under our experimental conditions, with any modification in the copper-binding capacity of the protein

Table 3
Statistical analysis of data using two-way analysis of variance

	Does albumin origin (BSA or HSA) affect the result?	Does glycoxidation treatment (G0/G5/G25 or MGO) affect the result?	Do both parameters exert any interactions?
Structural studies			
Thiol number	***	***	P=0.7284
Fluorescence	***	***	***
REM	***	***	***
Browning	***	***	***
Antioxidant properties			
HT_{50}	**	***	***
Copper binding	***	**	***
Effects on THP1 cells			
MTT measurements	P=0.2084	P=0.0546	P=0.5674
LDH measurements	**	P=0.2709	***
Cell counting	***	***	***
Proteasome LLVY	*	P=0.9285	**
Proteasome LSTR	***	P=0.0840	*
Proteasome LLE	*	***	**
Production of ROS	**	***	P=0.1198

Data in this study were obtained from a minimum of three independent experiments and were statistically analyzed using two-way analysis of variance (ANOVA). The major effects of the albumin origin (BSA or HSA) and glycoxidation treatment (GO, G5, G25, or MGO) or their interactions were evaluated by ANOVA. Proteasome LLVY, LSTR, and LLE correspond to the chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome, respectively. Significances were *** P<0.001, ** P<0.01, and * P<0.05. P values higher than 0.05 were specified.

(Fig. 3b). These results are in agreement with the previous observation showing that Cys34 blockage in HSA (using *N*-ethylmaleimide, NEM, reagent) was not associated with any reduction in the copper-binding capacity of the protein [30]. These results confirm that antioxidant activities exerted by albumin are somehow harbored by different localizations on the protein; therefore, these properties may differ even under same glycoxidation treatment. Although structural modifications after glycoxidation were more evident for BSA than for HSA, the impact of such treatment on the antioxidant properties of albumin was significant mainly for the human form of the protein.

Induction of oxidative stress by glycoxidized BSA or HSA on THP1 cells

Interaction of advanced glycoxidation end products with AGE receptors (RAGE) induced several cellular phenomena potentially relating to diabetic complications, including atherosclerosis. Monocyte activation, adhesion, and migration are the key events in this pathology. Conversely, AGE inhibitors reduced the expression of RAGE and other proinflammatory genes including monocyte chemoattractant protein-1 (MCP1), interferon γ-inducible protein-10 (INFγIP10), and cyclooxygenase-2 (Cox2) in a dose-dependent manner [19]. In addition, antioxidants inhibited the expression of the proinflammatory response in THP1 cells induced by the ligation of the receptor for AGEs [53]. Grune and his group, in one of their recent studies, showed that microglial cells were able to degrade glycoxidized proteins. Furthermore, an inhibition of the cellular proteolytic systems was demonstrated in the presence of AGEs in the culture medium [54]. Chronic exposure to glycoxidized serum in the J774.A macrophage cell line led to decreased expression of heat shock protein 70 (HSP 70) which became undetectable over time; this was explained on the basis of direct involvement of HSP 70 in the refolding of damaged proteins [55]. Furthermore, using the same cell type of macrophages, Davies's group showed that oxidized proteins altered the hydroperoxide phosphatase activity of cellular protein and the cellular redox signaling pathway [56]. Our team showed a pathophysiological effect of glycoxidized BSA on the primary cultures of human adipose cells by inducing an accumulation of oxidatively modified proteins [21]. More recently, several proteins particularly prone to carbonylation in cells incubated with AGEs were identified [22]. The heterogeneity of AGEs, experimental conditions under which they are generated, and concentrations used may lead to varied cellular responses. Valencia et al. showed that BSA incubated with 500 mM glucose failed to bind RAGE and to induce any cellular response when contaminating endotoxin was removed [57]. Our albumin preparations were analyzed for endotoxin content, which appeared below detectable levels. It should be noted that the majority of studies dealing with the influence of AGEs on cell physiopathology used either BSA or HSA modified by high non-physiological concentrations of glucose or MGO. Results reported here showed different and significant effects of low concentrations (10 μM) of mild modified albumin on the physiology of THP1 cells. In most instances, a drastic influence of modified albumin was noted for HSA_{MGO} and BSA_{MGO}. Interestingly, both HSA and BSA modified by physiological or pathological concentrations of glucose induced an enhanced cell proliferation as assayed by MTT measurement. Concerning cell counting, perplexing results were obtained after various treatments with glycoxidized albumin, as a reduced number of THP1 was counted when previously incubated with glycoxidized albumin. The MTT system was used for measuring the activity of living cells via mitochondrial dehydrogenases. One can hypothesize that the activity of this enzyme could be enhanced in glycoxidized albumin-treated cells. Otherwise, in the FACS-mediated cell countings, a window was defined to select and numerate only those cells presenting a turgescent and round shape. So we assume that the treatment of THP1 with AGEs modified the cell shape, leading to their displacement from the counting window. We could conclude that the presence of AGEs with mild concentrations used under these experimental conditions did not impair cell viability.

The oxidative modification of lipoproteins in the vascular wall leads to local production of reactive carbonyl species that mediate the recruitment of macrophages, cellular activation, and proliferation [58]. This multiplication of macrophages constitutes a typical feature of atherosclerosis. Conversely, the impact of AGEs on human mesenchymal stem cells inhibited the proliferation of cells, induced apoptosis, and prevented cognate differentiation into adipose tissue, cartilage, and bone [59]. Recent results showed that "rejuvenation" phenomenon in stem cells could be characterized by the decrease in AGEs and carbonylation associated with enhanced proteasomal activities in blastocytes on multiplication and differentiation [60].

A reduction in carbonylation of cell proteins was observed in those THP1 incubated in the presence of native albumin, showing the antioxidant protective effect of protein. Albumin glycoxidized with MGO induced an enhanced carbonylation of proteins in the cells. Glycoxidation of plasmatic proteins led to an altered cellular recognition and internalization of these particles, which were bound by the specific AGE receptors, named RAGE [61]. The RAGE-like receptor CD36 was overexpressed in aortic vascular smooth muscle cells treated with AGEs [62]. Here, elevations in the expressions of RAGE were especially noted in those cells incubated in the presence of glycoxidized BSA compared to native BSA. Surprisingly, a higher level of RAGE expression was observed in HSA_{MGO} and HSA_{GO}-treated cells. We hypothesize that the differential effects of BSA and HSA on human monocyte (THP1) RAGE expression might have a link with the difference in the origin of species for both the albumin and the cell used. Further investigations are necessary to detail the mechanisms of RAGE expression.

The proteasome represents the main constituent of the proteolytic pathway for the degradation of oxidized proteins [43]. Interestingly, enhanced chymotrypsin-like activity of the proteasome was observed in the cells incubated with glycoxidized albumin. Significant reduction in caspase-like activity was observed in HSA_{MGO}-treated cells. Impairments in the peptidylglutamyl-hydrolyzing activities of the proteasome were already reported when incubated with BSA_{G250} [54]. Definitely, it was reported that all three proteolytic activities were significantly reduced following the effects of 20 μg of BSA-AGE on 1 μg of isolated proteasome [54]. This concentration ratio between the oxidized compound (glycoxidized albumin) and the proteolytic system (proteasome) was high compared to the mild conditions used in our study. In addition, the concentrations of sugar used for BSA modifications were higher than 250 mM. It was also reported by the same group that exposure of proteasome to oxidized protein leads to a biphasic response in the proteolytic activities. At moderate oxidant concentrations, proteolytic susceptibility increases, whereas at higher oxidant concentrations, a decrease in proteolytic susceptibility occurs [63].

Antioxidant activity of native albumin in this cellular model of THP1 was further evidenced by the significant decrease in DCF fluorescence when cells were incubated in the presence of BSA_{GO} or HSA_{GO} as compared with the control (PBS). This protective action of albumin was significantly impaired after glycoxidation of the protein. Noteworthy, compared to BSA, HSA (glycoxidized or not) induced significant oxidative stress in THP1 cells, even though the carbonyl content is lower in HSA. Enhanced oxidative stress assayed by DCF-DA in macrophage-like RAW 264.7 cells exposed to various AGE albumin was reported by Subramaniam et al. [64]. In addition, they observed that the mere addition of unmodified albumin to cells was adequate to suppress the fluorescence of oxidized DCF. Here, the more significant results were again analyzed in the presence of MGO-modified albumin.

Oxidative modifications of albumin were observed when the protein was glycoxidized by pathophysiological concentrations of glucose. Such glycoxidized albumin induced an accumulation of carbonyl proteins and enhanced the formation of ROS. Impairments

in proteosomal activities in human monocytes were also observed. We demonstrate herein the important impact of the nature of albumin chosen in experiments dealing with structural/function relationships after the modification of the protein by glycoxidation. AGEs originating from the glycoxidation of BSA or HSA have lead to differential cellular responses. This study confirms and extends the determinant influence of the conditions in which AGEs are generated and concentrations used in experiments designed to mimic pathological conditions. Further ongoing projects try to better define the effects of AGEs on cellular physiology and to observe whether any impairment of proinflammatory cytokine expressions could be associated with the oxidative damages induced by glycoxidized albumin in cells.

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