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Identification of preferential protein targets for carbonylation in human mature adipocytes treated with native or glycated albumin

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Abstract

Oxidative modifications in proteins can participate in the regulation of cellular functions and are frequently observed in numerous states of diseases. Albumin can undergo increased glycation during diabetes. An accumulation of oxidatively modified proteins in human mature adipocytes incubated with glycated albumin has previously been described. This study herein reports the identification of specifically carbonylated targets following separation of the cell proteins by 2D gels, Western blotting and mass spectrometry analyses. It identified eight oxidatively modified proteins, two of which (ACTB and Annexin A2) appeared as significantly more carbonylated in adipocytes treated with glycated albumin than with native albumin. Intracellular stress, evaluated in SW872 cell line, showed an impairment in the protective antioxidant action exerted by native BSA after the glycation of the protein. Decreased proteasome peptidase activities were found in glycated BSA-treated mature adipocytes. The data suggest an association of oxidative damage with the progression of diabetes disorders at the adipocytes level.

Keywords: *Glycation, albumin, adipocytes, oxidative stress, oxidation, carbonyls*

Abbreviations: *ACTB, Actin Beta; AGEs, Advanced Glycated Endproducts; BSA, Bovine Serum Albumin; BSA_{GX}, BSA incubated with X mM of glucose; BSA_{G0}, BSA incubated in the absence of glucose; ECL, Enhanced Luminol Chemiluminescence; LDH, Lactate Deshydrogenase; MAD2, mitochondrial aldehyde dehydrogenase 2, OS; Oxidative Stress, PAGE, Polyacrylamide Gel Electrophoresis; RAGE, Receptor for Advanced Glycated Endproducts; ROS, Reactive Oxygen Species*

Introduction

Free radicals can interact with a variety of cellular components, altering both structure and function [1]. Oxidative modifications of proteins have been implicated in numerous disease states and more and more evidence indicates that these processes also participate in the regulation of cellular function [1–3].

Oxidative modifications of proteins could occur in non-insulin-dependent diabetes mellitus, which is one of the pathological conditions associated with early occurrence of vascular complications, together with functional alterations of albumin, which undergoes increased glycation [4,5]. The glycation phenomenon corresponds to the non-enzymatic attachment of a glucose molecule to a protein. Amadori rearrangement of the glycated protein gives rise to the deleterious advanced glycated end products (AGEs) [6]. The pathological conditions of diabetes with obesity have been widely associated

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with an enhanced oxidative stress [1,7]. Interaction of AGEs with their receptors (RAGE) induces several cellular phenomena potentially relating to diabetic complications. Recent data revealed that the binding of AGEs could be mediated by CD36, on adipose cells [8–10]. Since then, several studies have focused on the effects of AGEs on adipose cell physiology [11–13].

In a previous work, our group identified a pathophysiological effect of glycated BSA on primary cultures of human adipose cells by inducing an accumulation of oxidatively modified proteins [14]. The proteasome represents the main constituent of the proteolytic pathway for the degradation of oxidized proteins and is composed of at least three distinct peptidase activities: chymotrypsin-like, trypsin-like and caspase-like activities [15]. While the role of the proteasome in adipocytes is poorly understood, its activity may play an important role in controlling redox homeostasis and in degradating oxidized proteins [15–17]. Increasing evidence suggests that the activity of the proteasome can be altered under conditions of enhanced oxidative stress [15,18].

The present study was performed using proteomic analysis to identify proteins which can be carbonylated in human adipocytes incubated with native or glycated albumin. Immunochemical detection of protein carbonyls using 2D-oxyblot analysis coupled with peptide mass fingerprinting revealed several targets for protein oxidation. Eight proteins appeared as prone for carbonylation in adipocytes. Even if most of the eight identified proteins seemed more oxidized in glycated BSA-treated adipocytes, only annexin A2 and β Actin appeared, in our experimental conditions, to be significantly more oxidized in mature adipocytes treated with glycated albumin than with native albumin. Intracellular stress was evaluated in a cell line model of adipocytes (SW872). Reduction in oxidative stress exerted by native BSA was found altered after the glycation of the protein. Impaired proteasome peptidase activities were found in glycated BSA-treated mature adipocytes.

These novel findings support the association of oxidative damage with the progression of diabetic disorders at the adipocytes level.

Materials and methods

In vitro *glycation of BSA*

Non-defatted recombinant BSA (Sigma, cat # A2153) was dissolved in phosphate-buffered saline (PBS), pH 7.4, to yield a stock solution of 50 mg/mL. This solution was prepared with 200 mM glucose solution in PBS, to form incubation mixtures of 50 mg/mL BSA with 0 or 100 mM glucose. After being sterilized by filtration through 0.2 μM filters (Millipore), the solutions were incubated at 37°C for 50 days in capped vials. Reversible and unbound glucose were removed from BSA by extensive dialysis against PBS, pH 7.4. Samples were separated into aliquots and stored at −80°C in the dark prior to incubation with the adipocytes. Endotoxin content was below detectable level (0.03 Endotoxate unit/mL) as assessed with an *in vitro* toxicology assay kit (E-TOXATE, Sigma).

Cell cultures

Human liposarcoma cells (SW872) were obtained from Dr Lise Bernier's laboratory (Clinical Research Institute of Montreal). These adherent adipose cells were used in this work for oxidative stress measurements by DCF-DA assay using a micro-plate reader. Cells were seeded in six-multiwell plates and grown in Dulbecco's modified Eagle medium (DMEM) containing 1.25% L-glutamine, 2% penicillin/streptomycin and 10% foetal calf serum, in a humidified incubator (5% CO_2, 37°C).

Mature adipose cells were obtained from human subcutaneous adipose tissue. The procedures and the collection of human materials were approved by the local governmental French Ethical Committee. All adipose tissue donors were healthy women with normal glucose tolerance. Adipose tissues from the abdomen were rinsed in Ringer's solution and then submitted to the proteolytic action of collagenase at 1.5 mg/mL (NB4 Serva, Germany), during 25 min at 37°C under agitation. Digested tissue was filtered through a 80 μM membrane (Millipore) and submitted to a 30 s centrifugation at 1000 rpm, in order to separate mature adipocytes, which float in the supernatant. About 300 000 cells were seeded in a well of a 24-well plate containing 500 μl of DMEM (Sigma) medium, supplemented with 1.25% L-glutamine and 2% penicillin/streptomycin. Mature adipose cells were placed in a CO_2 incubator (5% CO_2/95% air) at 37°C. The incubation of the cells in the absence or the presence of the different BSA preparations was initiated at a maximum of 6 h after the adipose cells isolation.

Cell lysate preparation

After the incubation, the cells were washed in PBS. More precisely, as mature adipocytes are floating cells, they were first transferred to an eppendorf tube. Secondly, after a short spin at room temperature, the lower phase (medium) was removed and 1 mL of room-tempered PBS was added to the tube. This operation was repeated twice. Then, cells were treated at 4°C during 30 min with 100 μL of lysis buffer containing 25 mM Tris-HCl, pH 8.3, 10 mM KCl, 1 mM DTT, 1 mM EDTA, 1% Triton X100 and completed (excepted for the measurements of proteasomal activities) with a protease inhibitor cocktail (1% v/v, GE Healthcare). Cell lysates were then centrifuged at 15 000 rpm at 4°C for 20 min

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and the protein concentration was measured in the supernatant, using BCA metbod.

Two-dimensional gel electrophoresis

Prior to their separation by 2D gels, proteins were first labelled for their carbonyl content (Oxyblot Detection, Chemicon International Inc): 0.5 mg of proteins was denaturated by 12% SDS during 10 min at room-temperature. Samples were then treated with 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl, during 15 min at room temperature and neutralized.

The first dimension was performed utilizing Immobiline Drystrips (pH 3–10, length 13 cm) and the Multiphor II isoelectric focusing system (Amersham Pharmacia Biotech). About 0.5 mg protein from cell lysates was diluted in sample buffer (9 M urea, 2% Chaps, 2% Pharmalytes pH 3–10, 20 mM dithiothreitol and bromophenol blue). The Drystrip was rehydrated in this solution in a Re-swelling Tray (GE Healthcare) overnight at room temperature and then focused for 50 000 V-h (23 h). After focusing, the Immobilines Drystrips were equilibrated for 10 min in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 1% w/v SDS) supplemented with 1% (w/v) dithiothreitol followed by 10 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. The second dimension, SDS-PAGE, was performed using a 12% (w/v) gel and the Protean II electrophoresis system (Bio-Rad). For each 2D experiment, four 2D gels were run in parallel. Lysates from mature adipocytes isolated from three individuals were separated by 12 gels. After the migration, two gels were electro blotted onto nitrocellulose membrane and probed using antibody directed against the 2,4-dinitrophenol moiety (Oxyblot, Chemicon) and detection was performed using the ECL reagent (GE Healthcare). The two other 2D gels were stained with silver nitrate following established protocols [19].

Identification of oxidized proteins

Images generated from stained gels were overlaid with the results of the anti-DNPH analysis. Colocalizing spots were excised from the six gels and submitted separately for identification. Spots were sent to Innova Proteomics© (/www.innovaproteomics.com) for peptide mass fingerprinting identification by MALDI-TOF mass spectrometry.

Western blots

Proteins of mature adipocytes were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Biorad). Ponceau S Staining Solution (Sigma, P7170) was used for the detection of proteins on nitrocellulose membranes prior to immunological detections.

In the case of carbonyl detection, primary antibody used was directed against the 2,4-dinitrophenol moiety. The monoclonal antibodies JLA20 (anti-actin), EH7a (anti-annexin I and II) and 12G10 (anti α tubulin) were developed by Jim Jung-Ching Lin, Joel D. Ernst, Joseph Frankel and E. Marlo Nelsen, respectively. They were 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, IA. LMP2 constitutes a component of the eukaryotic multicatalytic proteinase complex, the proteasome. Polyclonal antibodies raised in rabbit against LMP2 sub-unit were obtained from Santa Cruz Biotechnology (Cat# SC28809).

After incubation with adequate HRP-coupled secondary antibody, detections were performed using the ECL reagent (GE Healthcare).

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

Proteasome activity measurements

Chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome were assayed using the fluorogenic peptides (from Sigma) U-Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA at 12.5 μM), N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-MCA at 12.5 μM) and N-Cbz-Leu-Leu-Glu-b-naphthylamide (LLE-NA at 12.5 μM), respectively [18]. Assays were carried out with $\sim$ 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 the samples was evaluated using a microplate spectrofluorometer reader (Fluostar–BMG France) at excitation/emission wavelengths of 350/440 and 333/410 nm for aminomethylcoumarin and b-naphthylamine products, respectively. Peptidase activities were measured in the absence or in the presence (10 μM) of the proteasome inhibitor MG132 (N-Cbz-Leu-Leu-leucinal) and the difference between the two values was attributed to proteasome activity.

Determination of ROS production

Human adipocytes (SW872 cell line) derived from a liposarcoma [20] were cultured in DMEM (Sigma) medium, supplemented with 10% foetal calf serum, 1.25% L-glutamine and 2% penicillin/streptomycin in a CO_2 incubator (5% CO_2/95% air) at 37°C. Four days before the experiment, 10^4 cells were plated in six well plates. Cells at $\sim$90% confluence were incubated in DMEM supplemented with 1.25% L-glutamine and 2% penicillin/streptomycin in the absence or presence of increasing concentrations of native or glycated BSA during 16 h. Cell medium

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was removed and replaced by 1 mL (per well) medium containing 10 μM final concentration of DCF-DA (Interchim) solubilized. After 30 min the fluorescence intensity of the oxidized form of DCF was measured in a microplate spectrofluorometer (Fluostar, BMG Labtech) at excitation and emission wavelengths of 492 nm and 520 nm, respectively.

Statistical analyses

Data are expressed as the means ± standard deviation (SD) from at least three experiments. Statistical significances were determined using Student's t-test.

Results

We recently observed after separation in one dimension of proteins from human mature adipocytes, a higher accumulation of carbonylated proteins in cells which were previously incubated for 16 h in the presence of 0.373 mM glycated BSA than with native BSA [14]. Some characterizations of structural modifications of BSA after glycation were previously performed by our group [5,14,21].

In this work, we aimed to evaluate which proteins in adipocytes can be subject to carbonylation and performed analyses of lysates from cells incubated with native or glycated albumin. Human mature adipocytes from three normoglycemic patients were treated with BSA_{G0} or BSA_{G100} during 16 h. Lysates were analysed on two-dimensional gels, separating by both charge and size. Carbonyl assays were performed to identify oxidative modifications [22].

We selected eight abundant carbonylated proteins that occupied distinctive location in the gel for identification by mass spectrometry. In Figure 1A and B, silver-stained 2D gels were performed from lysates of adipocytes which were incubated with native (BSA_{G0}) and glycated (BSA_{G100}), respectively. Proteins that appeared oxidized in carbonyl blots were numbered (1–8). Representative carbonyl blots performed from lysates of adipocytes which were incubated with native (BSA_{G0}) and glycated (BSA_{G100}) are shown in Figure 1C and D, respectively. Each of the protein spots that corresponded to oxidized proteins in the carbonyl blots was excised from the gel and subjected to MS/MS identification. In Table I, the proteins identified as subject to oxidation in mature human adipocytes were listed. The most dramatically glycated BSA-dependent oxidized proteins were Annexin A2 and ACTB. Signals for Annexin A2 and ACTB carbonylation (from blots) were quantified and expressed as amount of carbonyls per protein in Table II. The spots that correspond to Annexin A2 and to ACTB were

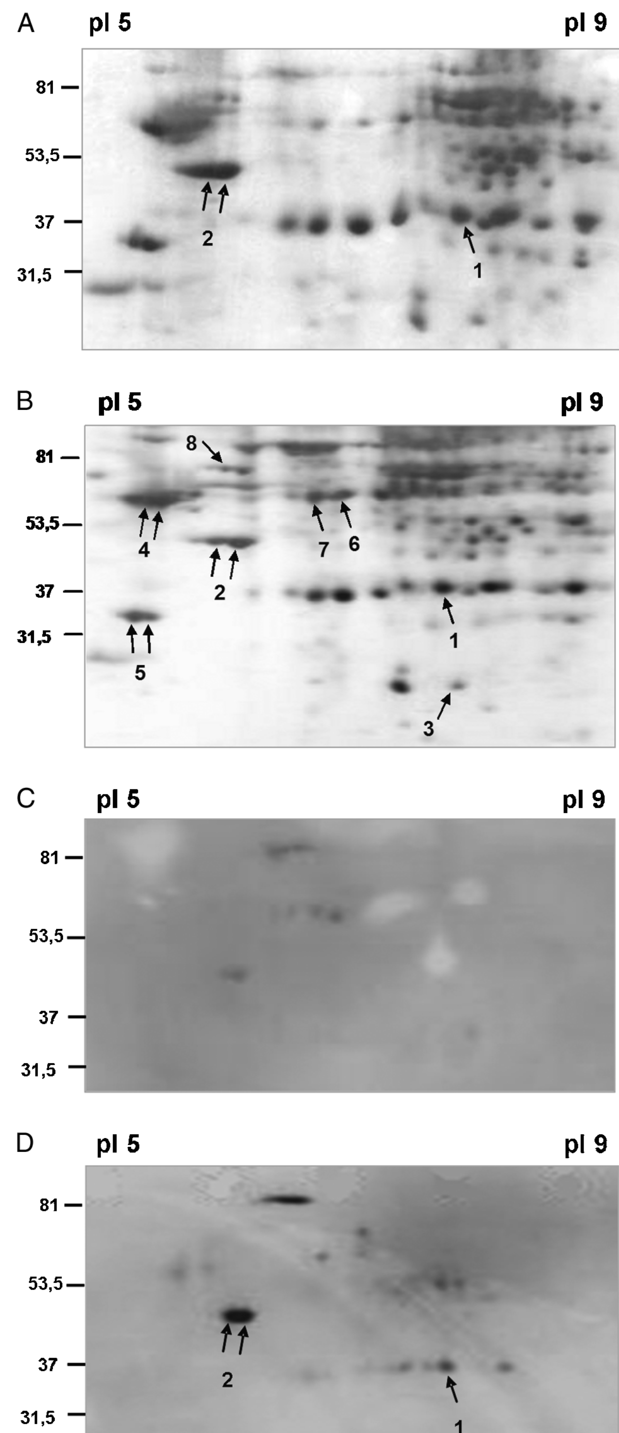

Figure 1. Many proteins are carbonylated in vitro in mature adipocytes. Human mature adipocytes from a primary culture were plated for 16 h in the presence of 0.37 mM BSA_{G0} or BSA_{G100}. Cells were harvested, lysed and cell proteins were derivatized with DNPH (see Materials and methods). Duplicate samples (~ 500 μg protein/gel) were separated on preparative 2D gels for either Silver staining (Gel A–sample BSA_{G0} and Gel B–sample BSA_{G100}), or anti-DNPH blots (Gel C–sample BSA_{G0} and Gel D–sample BSA_{G100}). Gel and blots shown represent protein of ~ 15–100 KDa and pI 5–9 (left to right). The photos shown are representative of one experiment that was repeated with similar results three times.

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Table I. Proteins identified as oxidized in human mature adipocytes. Proteins identified by mass spectrometry were listed according to the numbering shown in Figure 1. Accession numbers, molecular weight (KDa), isoelectric point and number of amino acids sequences vs total amino acid content were shown. Proteins identification was performed by Innova Proteomics™ and was determined from searching the human sub-set of the non-redundant NCBI database with peptide masses from the MALDI spectra. Coverage was calculated from the number of amino acid residues in the matched peptides, divided by the total number of residues in the protein. The probability-based Mowse score indicates the probability that the match between the database and a spectrum is a random event. This probability equals $10^{(-\text{Mowse score}/10)}$. Innova Proteomics™ established in their identification report that, in their analytical conditions, obtained scores of greater than 77 can be considered significant.

Spot #	Protein ID	Accession #	MW (kDa)/PI	Number peptides matched/searched	% coverage	Score
1	Annexin A2	16306978	38.8/7.6	10/18	38.5	134.5
2	ACTB	15277503	40.5/5.5	7/12	26.3	82.4
3	AFABP	52695842	14.7/7.6	7/19	44.5	86.5
4	ATP synthase	16741373	56.5/5.3	11/32	31.0	90
5	Annexin V	999937	35.8/5.0	9/23	35.5	102
6	Aldehyde dehydrogenase	178390	56.8/7	7/14	17.0	85
7	MAD2 precusor	62898307	56.8/6.6	7/13	20	88
8	Annexin VI isoform 1	71773329	76.2/5.4	10/34	24	77

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marked with an arrow in the two blots on Figure 1. These proteins were significantly more carbonylated in BSA_{G100} treated cells than in control ones (incubated with BSA_{G0}). Increases in carbonylation of Annexin A2 and ACTB were, when the mature adipocytes were incubated with BSA_{G100}, 575% and 373% ($p < 0.05$), respectively, in comparison with the control (BSA_{G0}).

Even if quantifications revealed no significant variations in the carbonyl content of the six other proteins, we noted in most cases a tendency for a higher oxidation in glycated BSA-treated cells than in native BSA-treated ones.

Total levels of ACTB did not change significantly depending on BSA_{G0} or BSA_{G100} treatment (Figure 2). Concerning Annexin A2, BSA_{G100} induced a slight decreased in the expression of the protein. The cell viability was estimated by measuring the Lactate Dehydrogenase (LDH) activity in the cell medium, using an in vitro toxicology assay kit and according to the manufacturer's instructions (Sigma, Cat # Tox7). No significant variation in LDH release in the medium was observed when mature adipocytes were incubated in the absence or the presence of native or glycated BSA (data not shown).

Table II. Annexin A2 and ACTB appeared markedly carbonylated in adipocytes treated with glycated albumin. Carbonyl contents in Annexin A2 and ACTB were determined from signal quantifications of scanned gels and films by using the freeware ImageJ. Values are expressed as mean ± SD of three different experiments (four 2D gels run per experiment). Values in arbitrary units represent mean ± SD of scans from three independent experiments. Statistical comparisons between values were performed using Student's t-test for unpaired samples ($n = 3$).

	Signal intensity ratio of carbonyl spot to identified protein – % increase vs CTL (BSA_{GO})	
	BSA_{GO}	BSA_{G100}
Annexin A2	100 ± 66	675 ± 350*
ACTB	100 ± 35	473 ± 166*

* $p < 0.05$.

It has been reported that pre-treatment of proteins with DNPH, which is necessary for immunodetection of reactive carbonyl groups, might affect the results of mass spectrometric identification of individual protein [23]. In our experimental conditions, protein spots were in most cases successfully analysed by mass spectrometry after excision from 2D-gels obtained with DNPH-treated protein samples. Noteworthy, the comparison between gel images obtained using the same sample, DNPH-treated or -untreated, showed some differences in protein position (Figure 3A). Conversely, Tezel et al. [23] observed no major difference in protein position after DNPH treatment. Additional identification of Annexin A2 and ACTB were conducted to validate peptide mass fingerprint results. Images in Figure 3B show Western blots performed after proteins separation on 2D-gels and using anti-Annexin A2 and ACTB antibodies.

Oxidative stress (OS) has been widely implicated in damage induced by biological compounds [1]. In an attempt to quantify OS in a cell model of adipocytes, we examined OS formed by incubating for 16 h with various concentrations of native or glycated BSA in SW872 cells by using the dichlorofluorescein diacetate (DCF-DA) assay [24]. After being internalized by the cell, hydrolysed (removal of acetate moiety) and after being oxidized by various oxidants, the non-fluorescent fluorescein derivatives (dichlorofluorescin, DCFH), will become DCF and emit fluorescence. These experiments were conducted in human liposarcoma cells (SW872) as they constitute adherent cells and allow the use of DCF assay in a multi-well plate reader [20,24]. Results of DCF assay were illustrated in Figure 4. Antioxidant property of BSA_{G0} was evidenced by the significant decrease in DCF fluorescence when cells were incubated in the presence of native BSA as compared with control (absence of BSA). This protective antioxidant activity

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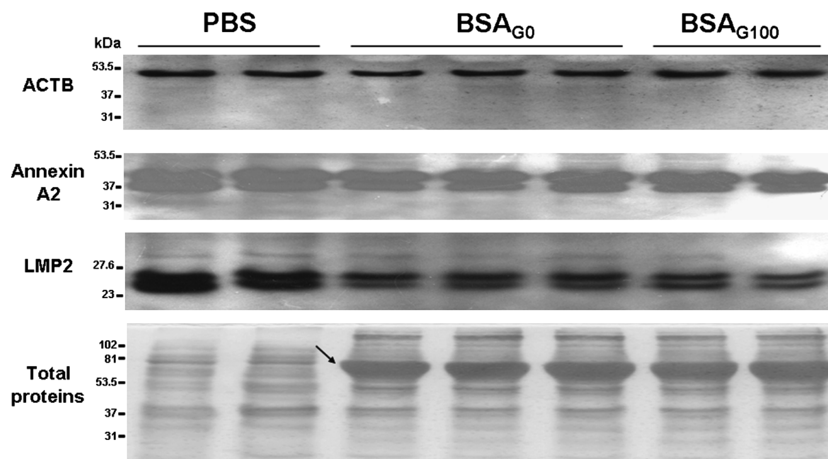

Figure 2. Effects of glycated BSA on the expressions of ACTB, Annexin A2 and LMP2 by adipocytes. Human mature adipocytes from a primary culture were plated for 16 h in the presence of 0.37 mM BSA$_{G0}$ or BSA$_{G100}$. About 25 μg of total protein from lysates of treated cells were separated by SDS-PAGE 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). Arrow on the gel indicates the position of BSA band.

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of BSA appeared at least slightly impaired after glycation of the protein as only a non-significant tendency for a decreasing in DCF fluorescence was noted when cells were incubated in the presence of BSA$_{G100}$. Enhanced oxidative stress assayed by DCF-DA in macrophage-like RAW 264.7 cells exposed to various AGE-albumins was reported [25]. In addition, Subramaniam et al. [25] observed that the mere addition of unmodified albumin to cells was sufficient to suppress the fluorescence of oxidized DCF.

The proteasome represents the main constituent of the proteolytic pathway for the degradation of oxidized proteins and is composed of at least three distinct peptidase activities: chymotrypsin-like, trypsin-like and caspase-like activities [15]. To better understand the origin of carbonylated proteins in BSA$_{G100}$-treated adipocytes, proteasome peptidase activities were measured in human mature adipocytes which have been incubated in the absence or the presence of native or glycated albumin. As shown in Figure 5, lowest peptidase activities were measured in a cell which had been incubated in the presence of BSA$_{G100}$. For all three peptidase activities, higher values were recorded when cells were incubated with native BSA or in the absence of BSA than in the presence of BSA$_{G100}$. The percentage reduction of the proteasome caspase-like activities measured in glycated BSA-treated cells vs native BSA-treated cells was -52% ($p < 0.05$). A slight but significant -9% decrease ($p < 0.05$ when compared with the control) was observed in the chymotrypsin-like activities in BSA$_{G100}$-treated cells. A non-significant reduction (-19%; $p = 0.07$ vs CTL) in the trypsin-like activities was noted when cells were incubated with glycated albumin. Recently, Stolzing et al. [26] reported an inhibitory effect of cross-linked protein-AGEs on proteolytic systems in microglial cells. As the loss of proteolytic activities could be related to a decrease in the proteasome levels, western blots were performed using antibodies directed against LMP2 sub-unit of the enzymatic complex (Figure 2). The proteasome expression appeared similar when cells were incubated with native or glycated BSA. This indicates that the impairment of the caspase-like activity in BSA$_{G100}$-treated cells might not be due to a decrease in the proteasome levels. Conversely, levels of LMP2 sub-unit seemed higher in control cells. This implies that adipocytes treated with BSA could lead to a reduced expression of the proteasome. Decrease in proteasome activity and content was reported by Pr. Friguet's group upon serial cell passaging [27]. Such impairments in proteasome were associated to increased levels of oxidized proteins, glycated proteins and proteins modified by the lipid peroxidation product 4-hydroxy-2-nonenal in the ageing cells.

Discussion

Obesity constitutes a principal causative factor in the development of metabolic syndrome. Increased oxidative stress was recently described in accumulated fat as an important pathogenic mechanism of obesity-associated metabolic syndrome [7]. Concept of redox control of cell homeostasis is relatively new and the recent onset of proteomics methods has allowed the oxidative stress response to be studied on large scales [28,29]. Identification of proteins subject to carbonylation in human mature adipocytes has never been investigated before. In this study, using two-dimensional gel technology and mass spectrometry, we have identified eight proteins in human mature adipocytes

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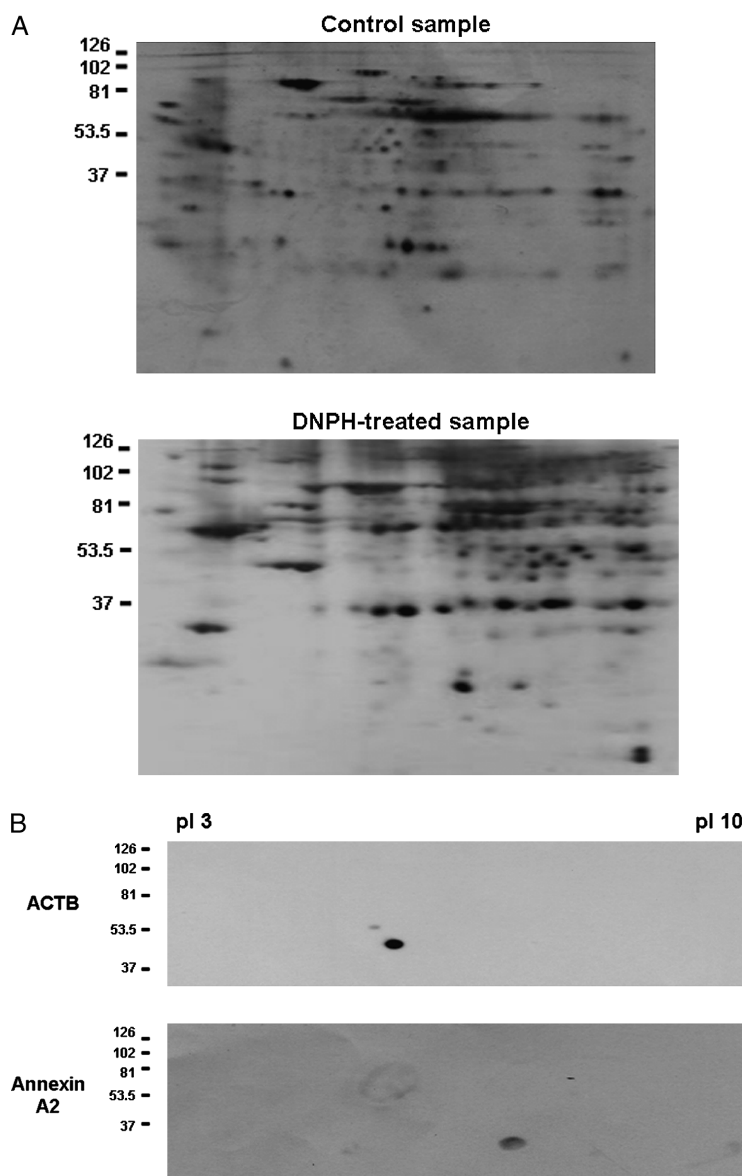

Figure 3. 2D-gels silver stained and identification of Annexin A2 and ACTB. Comparison between silver-stained gel images of the sample sample (A). DNPH-untreated (top) and DNPH-treated (bottom) samples showed no major differences in protein position. Western blots performed after separation of adipose proteins on 2D gels using anti-Annexin A2 and ACTB (B). Calculated PI and MW from these western blots were 38.8/7.6 and 40.5/5.5 for Annexin A2 and ACTB, respectively.

that were particularly prone to oxidative damage by carbonylation. In addition, two of these proteins, namely ACTB and annexin A2, acquired significantly more carbonyl modifications when cells were incubated in the presence of glycated albumin. All eight proteins were selected for mass spectrometric identification because they were clearly carbonylated and were both abundant and distinctively positioned in two-dimensional gels. The proteins identified fall into very few groups and in most instances a description of their crucial implication in adipocytes functioning can be discerned.

Three of the identified proteins belonged to the Annexin family, namely Annexin A2, A5 and A6. Annexins are ubiquitous, highly conserved, predominantly intracellular proteins and widely distributed in tissues. It was reported that oxidative stress could result in the glutathionylation of Cys 8 in Annexin A2. Moreover, Annexin A2 could constitute an oxidatively labile protein whose level of activity was regulated by the redox status of its sulphydryl groups [30]. In our experimental conditions, observed increases in carbonylation of Annexin A2 in BSA_{G100}-treated cells were associated with a reduced level of the protein (Figure 2). Converse observations were made by Tanaka et al. [31], who showed an increased level of Annexin A2 expression in cells exposed to H_2O_2. Carbonylated proteins are mostly degraded by the proteasome [15]. Here, all three proteasomal activities were lower in cells incubated with BSA_{G100}

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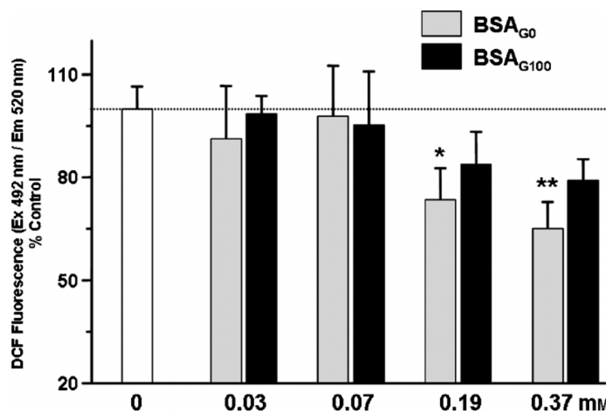

Figure 4. Impaired protective effect of BSA after glycation on free radical formation in SW872 cell line. The fluorescence intensity of the oxidized form of DCF was measured in SW872 cells which were incubated 16 h in absence (Control) or the presence of increasing concentrations of native or glycated BSA (for details please look to the Materials and methods section). The measurement of DCF fluorescence in the untreated cells was taken as 100%. The error bars represent the SD of the mean of three independent experiments. The significance of the results was determined by comparison with control values using one-way ANOVA, followed by Dunnett's test. * $p < 0.05$, ** $p < 0.01$.

than with native BSA, which may contributed to the accumulation of carbonylated ACTB and Annexin A2 in BSA_{G100}-treated cells. Little change and decrease in total amount of annexin A2 may come from the removal of the high oxidized protein fraction in BSA_{G100}-treated cells. One can hypothesize that in BSA_{G100}-treated cells, even if the proteasome was overwhelmed and somehow inhibited in the presence of a high amount of carbonylated protein, oxidized Annexin A2 and degradation may result in a noticeable decrease in the amount of the protein level.

Influence of inflammation on adipocytes metabolism was tightly associated to the increasing risk of developing obesity and metabolic syndrome [32,33]. Oxidative stress and carbonylated proteins were already linked to an abnormal coagulative pattern [34]. Here Annexin A5 was identified as prone to carbonylation in adipocytes. This protein represents a typical member of the annexin family, characterized by the ability to bind to phospholipid membranes in the presence of Ca^{2+}. Beneficial properties were reported for Annexin A5 due to its anti-thrombotic properties [35]. These were believed to be caused by its capacity to form a two-dimensional protective shield; covering exposed potentially thrombogenic cell surfaces [35].

Concerning annexin A6, several lines of experimental evidence suggested that the protein was involved in ion transport in various tissues [36]. Alteration by mutagenesis in Annexin sequence resulted in a defective formation of the GTP-induced

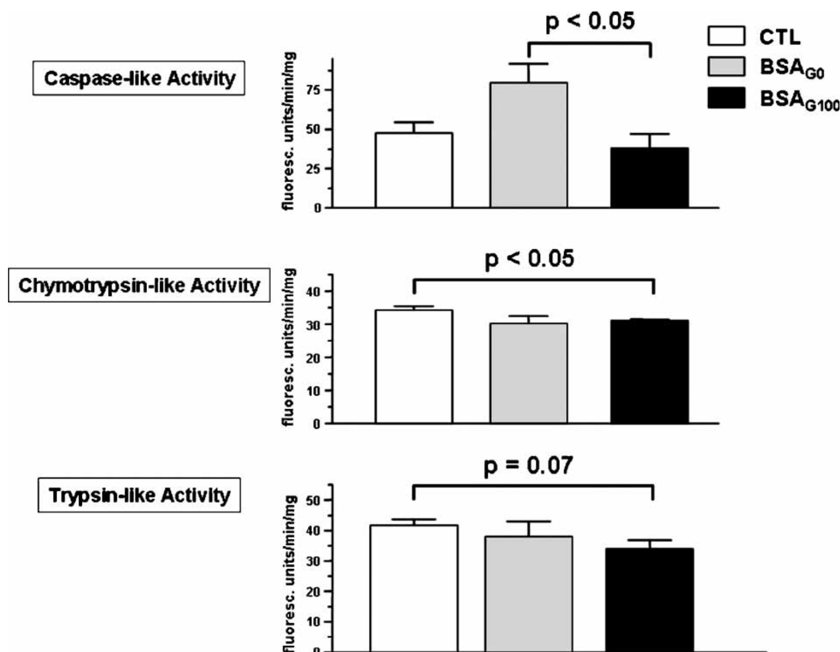

Figure 5. Effects of native or glycated BSA on proteasome function in mature adipocytes. Primary culture of human mature adipocytes was treated in the absence (CTL) or presence of 0.37 mM BSA_{G0} or BSA_{G100} for 16 h. Cells were washed and lysates were prepared as described in the Materials and methods section. The caspase-like, chymotrypsin-like and trypsin-like activities of the proteasome were assayed using the fluorogenic peptides substrates LLE-NA, LLVY-MCA and LSTR-MCA, respectively at 12.5 μM final concentration. Assays were carried out with 50 μg of cell lystes in 25 mM Tris-HCl (pH 7.5) and the appropriate substrate at 37°C for 0–30 min incubation. The fluorescence of the samples was evaluated using a multiplate spectrofluorometer (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 $\pm$SD ($n = 3$–8), p-values were obtained using Student's t-test.

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ion channels normally formed by trimeric association of annexin molecules [36]. Iron metabolism was tightly associated to oxidative modifications in protein and in a previous work 12 carbonylated proteins were identified in iron-treated cells [37].

Three out of the eight identified proteins prone to carbonyl modifications in adipocytes were enzymes: ATP synthase, aldehyde dehydrogenase and the mitochondrial aldehyde dehydrogenase 2 (MAD2) precursor. Interestingly enough, all three enzymes are mitochondrial proteins closely related to redox status in cells and an increasing number of reports supported their role as signalling molecules [38–41]. Mitochondrial reactive oxygen species generation was intimately associated with respiratory chain function [2]. The importance of mitochondria in adipocytes remained underestimated, however Carriere et al. [38] reported that mitochondrial oxidative metabolism was non-negligible in preadipocytes. Authors showed that rotenone and oligomycin, inhibitors of complex I and of ATP synthase, respectively, increased H_2O_2 and inhibited cell growth of preadipocytes, without inducing necrosis or apoptosis [38]. We think that, in our experimental conditions, observed oxidative alterations in these three mitochondrial proteins might be associated to weaker activities of the enzymes leading to a further enhancement of oxidative stress and damage in the adipocytes.

Another oxidized protein identified in adipocytes was the Adipocyte Fatty Acid Binding Protein (AFABP). The primary role of all the FABP family members is regulation of fatty acid uptake and intracellular transport [42]. AFABP is a plasma biomarker closely correlated with obesity and metabolic syndrome [43]. Annexin A2 and β actin (ACTB) were significantly more carbonylated in adipocytes incubated in the presence of glycated BSA than with native BSA. These two proteins were among the main endothelial plasmalemma-associated proteins forming glucose adducts in experimental diabetes [44]. Furthermore, very recent data demonstrated that actin dynamics in cells were regulated by Annexin A2 [45]. Recent data revealed that the binding of AGEs could be mediated by CD36 receptor on adipose cells [8–10]. Since then, several studies have focused on the effect of AGEs on adipose cell physiology [11,12,14,46]. In most cases, AGEs-treated cells elicited enhanced oxidative stress. Our present work reports for the first time a loss in the protective effect of native albumin on ROS production by adipocytes after the glycation of the protein. Previous works by our group showed the antioxidant activities of BSA were expressed both by binding capacity and free radical trapping property in the native form of the protein. Glycation and oxidation of the protein resulted in impaired structure and antioxidant properties of albumin [5,21,47].

Also, we have evidenced that AGEs-treated cells exerted inadequate proteasomal activities, unable to rid the adipocytes of damaged proteins. It is possible to better understand the origin of the accumulation of oxidized protein in AGEs-treated cells when one correlates this with an increased ROS production and an impaired proteasomal functioning. Preliminary observations by our group have indicated some impairment in proteasomal activities associated to a high ROS production in monocytes cell lines THP1 incubated with AGEs issued from both glycated HSA and BSA (unpublished results).

Works are under progress in our laboratory to determine if adipocytes treatment with native or glycated BSA results in a change in the association between ACTB and Annexin A2. Our present work suggests a mechanism by which certain proteins could be carbonylated in adipocytes treated with AGEs. AGEs accumulation constitutes a feature of metabolic syndrome. We propose that glycated albumin-dependent oxidation of a sub-set of proteins in adipocytes might be an unrecognized early event that leads to impairments in cell functioning. Further works will be needed to elucidate the role of AGEs-dependent oxidation at the adipocytes level in non-insulin-dependent diabetes mellitus.

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References

[1] Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.

[2] Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G, Szweda LI. Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during *in vivo* cardiac ischemia/reperfusion. Proc Natl Acad Sci USA 2005; 102:5987–5991.

[3] Sultana RPM, Butterfield DA. Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's disease: insights into mechanism of neurodegeneration from redox proteomics. Antioxid Redox Signal 2006;8:2021–2037.

[4] Cohen MP. Intervention strategies to prevent pathogenetic effects of glycated albumin. Arch Biochem Biophys 2003;419: 25–30.

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[5] Bourdon E, Loreau N, Blache D. Glucose and free radicals impair the antioxidant properties of serum albumin. Faseb J 1999;13:233–44.
[6] Cohen MP, Shea E, Chen S, Shearman CW. Glycated albumin increases oxidative stress, activates NF-kappa B and extracellular signal-regulated kinase (ERK), and stimulates ERK-dependent transforming growth factor-beta 1 production in macrophage RAW cells. J Lab Clin Med 2003; 141:242–249.
[7] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752–1761.
[8] Horiuchi S, Unno Y, Usui H, Shikata K, Takaki K, Koito W, Sakamoto Y, Nagai R, Makino K, Sasao A, Wada J, Makino H. Pathological roles of advanced glycation end product receptors SR-A and CD36. Ann N Y Acad Sci 2004; 1043:671–675.
[9] Horiuchi S, Sakamoto Y, Sakai M. Scavenger receptors for oxidized and glycated proteins. Amino Acids 2003;25:283–292.
[10] Kuniyasu A, Ohgami N, Hayashi S, Miyazaki A, Horiuchi S, Nakayama H. CD36-mediated endocytic uptake of advanced glycation end products (AGE) in mouse 3T3-L1 and human subcutaneous adipocytes. FEBS Lett 2003;537:85–90.
[11] Uchida Y, Ohba K, Yoshioka T, Irie K, Muraki T, Maru Y. Cellular carbonyl stress enhances the expression of plasminogen activator inhibitor-1 in rat white adipocytes via reactive oxygen species-dependent pathway. J Biol Chem 2004;279:4075–4083.
[12] Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M, Geloen A. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. Free Radic Biol Med 2005;38:882–889.
[13] Unno Y, Sakai M, Sakamoto Y, Kuniyasu A, Nakayama H, Nagai R, Horiuchi S. Advanced glycation end products-modified proteins and oxidized LDL mediate down-regulation of leptin in mouse adipocytes via CD36. Biochem Biophys Res Commun 2004;325:151–156.
[14] Chesne S, Rondeau P, Armenta S, Bourdon E. Effects of oxidative modifications induced by the glycation of bovine serum albumin on its structure and on cultured adipose cells. Biochimie 2006;88:1467–1477.
[15] Friguet B. Oxidized protein degradation and repair in ageing and oxidative stress. FEBS Lett 2006;580:2910–2916.
[16] Xu GSC, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, Londos C. Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. J Biol Chem 2005;52:42841–42847.
[17] Rui LFT, Thomas J, White MF. Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J Biol Chem 2001;43:40362–40367.
[18] Le Pecheur M, Bourdon E, Paly E, Farout L, Friguet B, London J. Oxidized SOD1 alters proteasome activities in vitro and in the cortex of SOD1 overexpressing mice. FEBS Lett 2005;579:3613–3618.
[19] Shevchenko AWM, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–858.
[20] Wassef H, Bernier L, Davignon J, Cohn JS. Synthesis and secretion of apoC-I and apoE during maturation of human SW872 liposarcoma cells. J Nutr 2004;134:2935–2941.
[21] Rondeau P, Armenta S, Caillens H, Chesne S, Bourdon E. Assessment of temperature effects on beta-aggregation of native and glycated albumin by FTIR spectroscopy and PAGE: relations between structural changes and antioxidant properties. Arch Biochem Biophys 2007;460:141–150.
[22] Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346–357.
[23] Tezel G, Yang X, Cai J. Proteomic identification of oxidatively modified retinal proteins in a chronic pressure-induced rat model of glaucoma. Invest Ophthalmol Vis Sci 2005;46:3177–3187.
[24] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27:612–616.
[25] Subramaniam R, Fan XJ, Scivittaro V, Yang J, Ha CE, Petersen CE, Surewicz WK, Bhagavan NV, Weiss MF, Monnier VM. Cellular oxidant stress and advanced glycation endproducts of albumin: caveats of the dichlorofluorescein assay. Arch Biochem Biophys 2002;400:15–25.
[26] Stolzing A, Widmer R, Jung T, Voss P, Grune T. Degradation of glycated bovine serum albumin in microglial cells. Free Radic Biol Med 2006;40:1017–1027.
[27] Petropoulos I, Conconi M, Wang X, Hoenel B, Bregegere F, Milner Y, Friguet B. Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells. J Gerontol A Biol Sci Med Sci 2000;55:B220–B227.
[28] Rabilloud T, Chevallet M, Luche S, Leize-Wagner E. Oxidative stress response: a proteomic view. Expert Revi Proteomics 2005;2:949–956.
[29] Rottoli P, Magi B, Cianti R, Bargagli E, Vagaggini C, Nikiforakis N, Pallini V, Bini L. Carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. Proteomics 2005;5:2612–2618.
[30] Caplan JF, Filipenko NR, Fitzpatrick SL, Waisman DM. Regulation of annexin A2 by reversible glutathionylation. J Biol Chem 2004;279:7740–7750.
[31] Tanaka T, Akatsuka S, Ozeki M, Shirase T, Hiai H, Toyokuni S. Redox regulation of annexin 2 and its implications for oxidative stress-induced renal carcinogenesis and metastasis. Oncogene 2004;23:3980–3989.
[32] Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006;116:1793–1801.
[33] Galinier A, Carriere A, Fernandez Y, Caspar-Bauguil S, Periquet B, Periquet A, Penicaud L, Casteilla L. Site specific changes of redox metabolism in adipose tissue of obese Zucker rats. FEBS Lett 2006;580:6391–6398.
[34] De Cristofaro R, Rocca B, Vitacolonna E, Falco A, Marchesani P, Ciabattoni G, Landolfi R, Patrono C, Davi G. Lipid and protein oxidation contribute to a prothrombotic state in patients with type 2 diabetes mellitus. J Thromb Haemost 2003;1:250–256.
[35] Cederholm A, Frostegard J. Annexin A5 in cardiovascular disease and systemic lupus erythematosus. Immunobiology 2005;210:761–768.
[36] Kirilenko A, Pikula S, Bandorowicz-Pikula J. Effects of mutagenesis of W343 in human annexin A6 isoform 1 on its interaction with GTP: nucleotide-induced oligomer formation and ion channel activity. Biochemistry 2006;45: 4965–4973.
[37] Drake SK, Bourdon E, Wehr NB, Levine RL, Backlund PS, Yergey AL, Rouault TA. Numerous proteins in Mammalian cells are prone to iron-dependent oxidation and proteasomal degradation. Dev Neurosci 2002;24:114–124.
[38] Carriere A, Fernandez Y, Rigoulet M, Penicaud L, Casteilla L. Inhibition of preadipocyte proliferation by mitochondrial reactive oxygen species. FEBS Lett 2003;550:163–167.
[39] Demozay D, Rocchi S, Mas J, Grillo S, Pirola L, Chavey C, Van Obberghen E. Fatty aldehyde dehydrogenase: potential role in oxidative stress protection and regulation of its

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gene expression by insulin. J Biol Chem 2004;279:6261–6270.

[40] Wenzel P, Hink U, Oelze M, Schuppan S, Schaeuble K, Schildknecht S, Ho KK, Weiner H, Bachschmid M, Munzel T, Daiber A. Role of reduced lipoic acid in the redox regulation of mitochondrial aldehyde dehydrogenase (ALDH-2) activity: implications for mitochondrial oxidative stress and nitrate tolerance. J Biol Chem 2007;282:792–799.

[41] Deveaud C, Beauvoit B, Salin B, Schaeffer J, Rigoulet M. Regional differences in oxidative capacity of rat white adipose tissue are linked to the mitochondrial content of mature adipocytes. Mol Cell Biochem 2004;267:157–166.

[42] Chmurzynska A. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. J Appl Genet 2006;47:39–48.

[43] Xu A, Wang Y, Xu J, Stejskal D, Tam S, Zhang J, Wat N, Wong W, Lam K. Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. Clin Chem 2006;52:405–413.

[44] Ghitescu LD, Gugliucci A, Dumas F. Actin and annexins I and II are among the main endothelial plasmalemma-associated proteins forming early glucose adducts in experimental diabetes. Diabetes 2001;50:1666–1674.

[45] Hayes MJ, Shao D, Bailly M, Moss SE. Regulation of actin dynamics by annexin 2. Embo J 2006;25:1816–1826.

[46] Unno Y, Sakai M, Sakamoto Y, Kuniyasu A, Nagai R, Nakayama H, Horiuchi S. Glycolaldehyde-modified bovine serum albumin downregulates leptin expression in mouse adipocytes via a CD36-mediated pathway. Ann N Y Acad Sci 2005;1043:696–701.

[47] Bourdon E, Loreau N, Lagrost L, Blache D. Differential effects of cysteine and methionine residues in the antioxidant activity of human serum albumin. Free Radic Res 2005; 39:15–20.

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