

# Identification of Up-Regulated Low Molecular Weight Proteins in Human Adipocytes Treated with Glycoxidized Albumin

Nihar Ranjan Singh, Philippe Rondeau and Emmanuel Bourdon\*

Laboratoire de Biochimie et Génétique Moléculaire (LBGM), Groupe d'Etude sur l'Inflammation Chronique et l'Obésité (GEICO), Université de La Réunion, Saint Denis de La Réunion, France

**Abstract:** Diabetes and Obesity are strongly associated with enhanced circulating advanced glycoxidation end products (AGEs), and adipose tissue constitutes a biological driver in the metabolic syndrome characterized by abdominal obesity, dyslipidemia and insulin resistance. We determined whether AGEs issued from glycoxidized albumin could induce expression of determinant proteins in human adipocyte cell lines (SW872).

Cell proteins separation by 2D gels and mass spectrometry were combined to identify up-regulated proteins in SW872 treated with AGEs. Four low molecular weight proteins appeared as more expressed in adipocytes treated with glycated albumin than with native albumin. All four proteins have determinant role in adipocyte physiology as they exert antioxidant activities (superoxide dismutase and proteasome subunit) or energy production properties (phosphoglycerate mutase and triosephosphate isomerase).

Proteomic analyses of SW872 cells have never been performed before. By using proteomic analyses, our results bring new evidence of AGEs-involvement in metabolic disorders at the adipocyte level.

**Keywords:** Glycoxidation, albumin, adipocytes, oxidative stress, SW872, glycation, diabetes.

## 1. INTRODUCTION

It is now well established that free radicals and reactive oxygen species (ROS) contribute to the development of several age-related diseases by inducing oxidative stress and oxidative damages [1]. The incidence of diabetes is increasing, with a worldwide prevalence estimated to double by 2030, primarily because of sedentary lifestyle and obesity [2]. A strong link between obesity and diabetes is now well established with more than 70 percent of people with diabetes being overweight. This disease, which is characterized by hyperglycemia and insulin resistance, is closely associated with severe complications. Indeed, diabetes increases the risk of developing cardiovascular disease, which represents the leading cause of mortality in western countries [3]. Recently, a causal role was shown for ROS in multiple forms of insulin resistance, which was also associated with elevated formation of advanced glycation end products (AGEs) [4]. Adipocytes are known to express and secrete a variety of active molecules, so-called adipokines, which can regulate many biological processes such as insulin-sensitivity, appetite, immunity, reproduction. It is now well established that dysregulation of adipocyte functions by oxidative stress constitutes a key process leading to type 2 diabetes. It is thus not surprising that many diabetic therapies target adipose tissue (thiazolidinedione, physical activity, etc) [5,6]. SW872 cells constitute a liposarcoma cell line often used as a human adipocyte cell model [7-12]. Nonetheless, proteomic analyses of SW872 cells have never been performed before. In

this work, we determined whether AGEs issued from glycoxidized albumin could induce expression of determinant proteins in human adipocyte cell lines (SW872).

## 2. MATERIALS AND METHODS

### *In Vitro* Glycoxidation 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<sup>-1</sup>. This solution was prepared with 200 mM glucose solution in PBS, to form incubation mixtures of 50 mg.mL<sup>-1</sup> 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. Albumin preparations were assessed for glucose content after dialyses and the later was below detectable levels. Samples were separated into aliquots and stored at -80 °C in the dark prior to incubation with the SW872 cells. Endotoxin content was below detectable level (0.03 Endotoxate Unit/mL) as assessed with an in vitro toxicology assay kit (E-TOXATE, Sigma).

### Mass Spectrometry Studies of Native and Glycoxidized BSA

Native BSA and glycoxidized BSA treated in the absence or the presence of 100 mM of glucose, were analyzed using mass spectrometry for molecular weight and extent of glycoxidation determinations. ESI/MS was performed on Agilent 1100LC coupled to Bruker Esquire 3000plus operating in the positive ion mode as mentioned earlier [20].

\*Address correspondence to this author at the LBGM - Université de La Réunion - 15, avenue René Cassin - BP 7151 - Cedex 09, 97715 Saint Denis de La Réunion, France; Tel: (+262) 262 93 86 48; Fax: (+262) 262 93 82 37; E-mail: emmanuel.bourdon@univ-reunion.fr

## Cell Culture

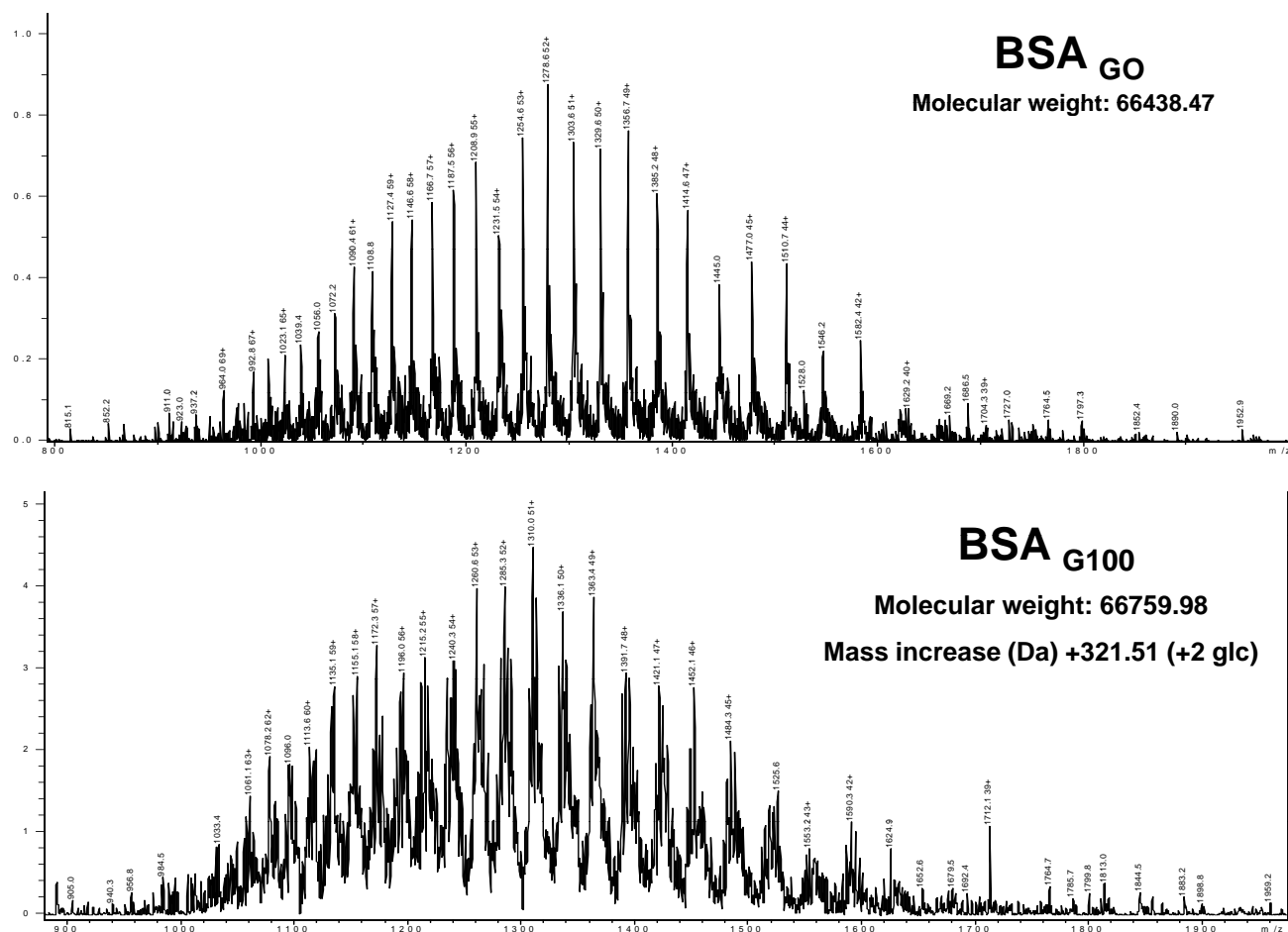
Human SW872 liposarcoma cells were purchased from American Type Culture Collection. Cells were seeded in six-multiwell plates and grown in Dulbecco's modified Eagle medium (DMEM) containing 1.25% L-glutamine, 2% penicillin/streptomycin, and 10% fetal calf serum, in a humidified incubator (5% CO<sub>2</sub>, 37°C). The culture plates were incubated until cells reach 75% confluency before adding BSA<sub>G0</sub> or BSA<sub>G100</sub> at a final concentration of 20 μM. Thereafter, cells were maintained in the humidified CO<sub>2</sub> incubator for 16 h before further analyses.

## Cell Lysate Preparation

After the incubation, the cells were washed twice with PBS. 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. Cell lysates were then centrifuged at 15,000 rpm at 4°C for 20 min and the protein concentration were measured in the supernatant.

## Two-Dimensional Gel Electrophoresis

The first dimension was performed utilizing Immobiline Drystrips (pH 6.2 -7.5, length 7 cm) and the Multiphor II isoelectric focusing system (Amersham Pharmacia Biotech). 0.5 mg protein from cell lysates was diluted in sample buffer (9 M urea, 2% Chaps, 2% Pharmalytes, 20 mM dithiothreitol, and bromophenol blue). Lysates from SW872 cells treated with native and glycated albumin were isolated and separated on Immobiline Drystrips. The Drystrip was rehydrated in this solution in a re-swelling tray (GE Healthcare) overnight at room temperature and then focused for 50,000 Vxh<sup>-1</sup> (23 h). After focusing, the Immobiline 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, two strips of 7 cm each were run side by side on the same gel. This experiment was replicated four times for consistency in



**Fig. (1).** Mass spectrometry analysis of native and glycoxidized BSA.

Native BSA and glycoxidized BSA treated in the absence or the presence of 100 mM of glucose, were analyzed using mass spectrometry for molecular weight and extent of glycoxidation determinations. ESI/MS was performed on Agilent 1100LC coupled to Bruker Esquire 3000plus operating in the positive ion mode as mentioned in [20].

results. These 2D gels were stained with silver nitrate following established protocols [13].

### Identification of Proteins

Images generated from silver stained gels were compared. Co-localizing spots were excised from the silver stained gels and submitted separately for identification. Spots were sent for peptide mass fingerprinting identification by MALDI-TOF/TOF mass spectrometry, at the Plateforme Technologique de Biotechnologie Moléculaire (Université d'Angers, France). Signal quantifications from images were determined using the freeware ImageJ (version 1.32j), available from the internet website: <http://rsb.info.nih.gov/ij/>.

## 3. RESULTS

### Mass Spectrometry Analysis of Native and Glycoxidized Albumin

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 diabetes, albumin undergoes increased glycation and glycoxidation [14]. The glycation phenomenon corresponds to the non-enzymatic and non-oxidative covalent attachment of glucose molecule to protein [14]. Glycoxidation refers to the radical-mediated oxidation reaction of both free and protein-bound sugars [15]. Amadori rearrangement of the above glycated protein gives rise to deleterious, advanced glycoxidation (also termed advanced glycation) end products (AGEs) [16,17].

Modifications of commercial BSA when incubated with 0 mM and 100 mM of glucose were assessed by ESI/MS, which indicate the number of glucose molecule condensed on the protein. The most abundant component of native and glycoxidized albumin is shown in Fig. (1). Additional important characterizations of structural modifications in HSA and

BSA after incubating with varied concentration of glucose were previously performed by our group [18-20].

As expected, non-enzymatic glycosylation of BSA induces an increase in molecular weight for albumin incubated with 100 mM of glucose, in comparison with native BSA. Here, the molecular mass represented by the most abundant component of non-modified BSA and glycoxidized albumin is 66438.47 Da and 66759.98 Da, respectively. This increase of 321.51 Da could correspond to the addition of two molecules of glucose unit to the glycoxidized albumin.

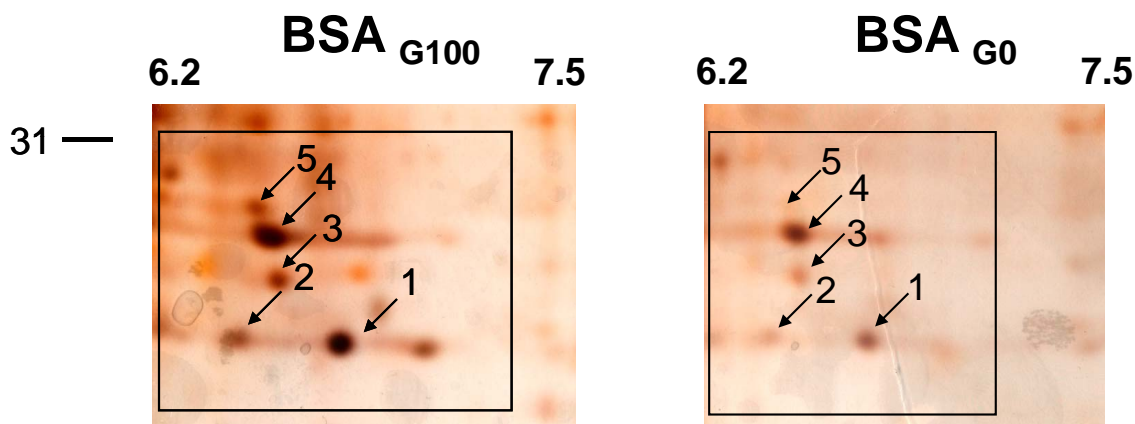
### Protein Expression in SW872 Cells after Incubation with Native or Glycoxidized Albumin

Expression of up-regulated and/or down-regulated proteins in SW872 cell was analyzed on silver stained 2D gel after adipocyte incubation with native and glycoxidized albumin. The protein spots were excised from silver stained 2D gel, which separates the proteins by both charge and size. We selected 5 proteins spots that occupy the low molecular weight region. Fig. (2) represents the 2D gel in which the low molecular weight proteins expression levels are compared when SW872 cells were incubated with native or glycoxidized albumin. Proteins identified by mass spectrometry are listed according to the number on the silver stained gel as shown in Fig. (2).

In Table 1, proteins are identified using MALDI TOF/TOF apparatus from the silver stained 2D gels. Identified proteins show higher expression level in BSA<sub>G100</sub>-treated cells. The proteins identified fall into few groups and in most instances a description of their crucial implication in adipocyte functioning can be discerned.

## 4. DISCUSSION

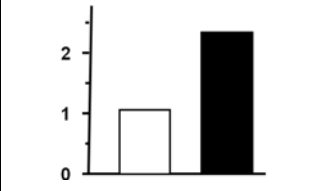
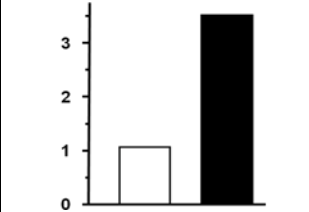
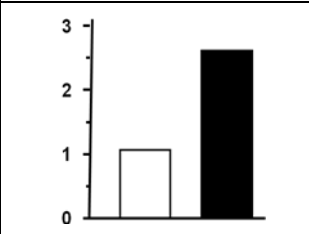
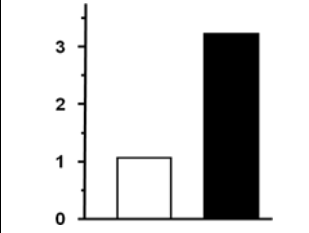
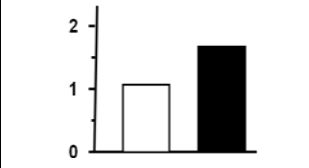
Diabetes and obesity constitute the major causative factor that can lead to the development of metabolic syndrome. Elevated concentration of glucose induces non-enzymatic glycosylation of albumin altering both the structure and the



**Fig. (2). Five low molecular weight proteins are higher expressed in AGEs-treated adipocytes.**

SW872 cells were incubated for 16hrs in the presence of 20  $\mu$ M of BSA<sub>G100</sub> or BSA<sub>G0</sub>. Cells were harvested, lysed and cell proteins were derivatized with DNPH (see Materials and methods). Samples (500 mg protein/gel) were separated on preparative 2D gels for Silver staining. Gel shown represents protein of less than 30 KDa and pI 6.2-7.5 (left to right). The photos shown are representative of one experiment that was repeated with similar results three times.

**Table 1. Differentially Accumulated Proteins and their Identification by Mass Spectrometry were Listed According to the Numbering Shown in Fig. (2). Accession Numbers, Theoretical Molecular Weight (kDa) and Isoelectric Point, Number of Amino Acid Sequence Matched and Percentage Identification Score were Shown. Protein Identification was Performed at University of Angers and was Determined by Searching the Human Sub-Set of the Non-Redundant NCBI Database with Peptide Masses from MALDI Spectra**

Spot #	Protein ID	Accession #	MW kDa/ PI	Number of Peptides Matched	% Identification Score	Spot Pattern (Relative Spot Volume) □ BSA <sub>G0</sub> ■ BSA <sub>G100</sub>
1	Superoxide dismutase [Mn]	P04179	22.2/ 6.86	1	98	
2	Superoxide dismutase [Mn]	P04179	22.2/ 6.86	2	60	
3	Proteasome subunit alpha type 2	P25787	25.7/7.12	2	97	
4	Phospho-glycerate mutase 1	P18669	28.6/6.75	3	99	
5	Triosephos-phate isomerase	P60174	26.5/6.51	6	99	

functions of this crucial plasmatic protein [21,22]. The current study was undertaken to identify dysregulated proteins in human adipocytes submitted to glycated albumin. SW872 cell line, which constitutes a good model for adipocytes, was used to analyze the expression level of proteins. In total, four low molecular proteins were selected for mass spectrometric identification because they were clearly over expressed in glycated BSA-treated adipocytes and distinctly positioned on two- dimensional gel. The proteins fall into few groups and in most instances a description of their crucial implication in SW872 functioning can be established.

Identified proteins belonged to different groups that include an antioxidant enzymatic system, degradation pathway and glycolysis pathway. Mitochondrial superoxide dismutase (MnSOD) is the major antioxidant protein found to be highly

expressed in SW872 cells that were incubated with glycoxidized albumin compared to native albumin. MnSOD is manganese containing protein, which belongs to a large family of metalloproteins. The SOD protein family participates in scavenging superoxide radicals generated mainly by the electron transport system in mitochondria. High levels of MnSOD in SW872 cells incubated with glycoxidized albumin may be due to an enhanced synthesis of the enzyme in response to the oxidative stress. Yoo et al. have shown that glycated albumin induces superoxide generation in mesangial cells [23]. Very recently, enhanced ROS generation was evidenced in erythrocytes issued from Cu/Zn SOD knock out mice [24]. A positive correlation was established between mitochondrial hydrogen peroxide generation, MnSOD activity and oxidative damages in lipids and proteins [25].

Similarly, there is a 3 fold increase in the expression levels of proteasomal subunit  $\alpha 2$ . Proteasome is a multi protein complex that is known to degrade altered protein. The 20S subunit of proteasome consists of two outer rings containing seven different  $\alpha$ -subunits ( $\alpha 1$ - $\alpha 7$ ) and two middle central rings each is constituted by seven different  $\beta$ -subunits ( $\beta 1$ - $\beta 7$ ). 20S subunit is involved in the degradation of oxidized proteins [26]. Reports from our group showed that enhanced oxidative stress and oxidative damages in AGEs-treated cells were associated with impairments in the proteasomal activities [7,20]. Increased expression level of proteasome in SW872 cells are consistent with very recent data from Schimdt et al who evidenced induction of heat shock proteins and the proteasome system by two different types of AGEs in Caco-2 cells [27].

Phosphoglycerate mutase 1 and triosephosphate isomerase proteins also appeared to accumulate more importantly in SW872 cells that were incubated with BSA<sub>G100</sub> than with BSA<sub>G0</sub>. These enzymes are closely involved in the glycolysis pathway, where the glucose is oxidized to produce energy. These enzymes are closely related to the insulin sensibility in adipocytes, which constitutes a emerging issue in the obesity-associated diabetes pathology [28].

This study warrants further investigation. Nonetheless, we propose that AGEs-mediated enhancement in the accumulation of these four proteins in adipocytes may be involved in the adipose tissue inflammation inherent to obesity-associated disorders.

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## ABBREVIATIONS

AGEs	=	Advanced Glycated Endproducts
BSA	=	Bovine Serum Albumin
BSA <sub>GX</sub>	=	BSA incubated with x mM of glucose
BSA <sub>G0</sub>	=	BSA incubated without glucose
OS	=	Oxidative Stress
PAGE	=	Polyacrylamide Gel Electrophoresis
ROS	=	Reactive Oxygen Species

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