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Aspirin-mediated acetylation of haemoglobin increases in presence of high glucose concentration and decreases protein glycation

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ABSTRACT

Glycation represents the first stage in the development of diabetic complications. Aspirin was shown to prevent sugars reacting with proteins, but the exact mechanism of this interaction was not well defined. We performed a quantitative analysis to calculate the levels of acetylation and glycation of haemoglobin, among others red blood cell (RBC) proteins, using a label free approach. After glucose incubation, increases in the acetylation levels were seen for several haemoglobin subunits, while a parallel decrease of their glycation levels was observed after aspirin incubation. These results suggest that, a mutual influence between these two modifications, occur at protein level.

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1. Introduction

As diabetes progresses, the number of disease-specific complications has a tendency to increase. Among other risk factors, this is mostly due to chronic hyperglycaemia which promotes an uncontrolled, non-enzymatic modification of proteins called glycation. This post-translational modification (PTM) occurs between electrophilic glucose and nucleophilic primary amino groups of proteins (typically the N-terminal and ε amines of lysine) to generate a stable covalently bound intermediate, the Amadori compound. The latter represents the central core for the subsequent irreversible reactions of the glycation process [1]. The reaction's kinetics are enhanced by high and prolonged exposure to glucose, which in turn leads to the chronic health problems commonly observed in diabetes, including neuropathy [2], nephropathy [3], retinopathy [4] and cardiovascular diseases [5,6]. These disorders frequently appear several years after the onset of the illness, therefore, timely glycaemic control is of utmost importance in order to minimize the deleterious effects of glucose [7]. Today, blood glucose is typically monitored using the glycated haemoglobin (HbA1c) test considered, the gold standard for disease definition and the monitoring of anti-diabetes treatment [8]. Nevertheless, increasing evidence has shown that glycated haemoglobin may be affected by different inter-individual

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factors, leading to a "glycation gap" between the HbA1c and the level of mean plasma glucose [9,10]. This discrepancy can result from any one of a range of physiological variables that leads to an underestimate of the HbA1c levels in diabetic patients. These include any disorder that alters the lifespan of erythrocytes (red blood cells, RBCs) such as renal failure, haemolytic anaemia, iron-deficiency anaemia or a blood transfusion. Moreover, haemoglobin variants and adducts, which result from PTMs of haemoglobin (carbamylated Hb and pre-HbA1c), may alter the outcome of the test, thus generating method-specific interferences [11]. It should be noted that haemoglobin is the most abundant protein in RBCs, and the variation of its glycation levels is mostly due to its continuous interaction with circulating glucose, which also, influences other RBC proteins. As a consequence, hyperglycaemia has deleterious effects on the lifespan of RBCs, altering deformability and oxidative stress, which in turn lead to the hypertension and vascular complications [12–16] typical of diabetes.

Over the last few decades, it has been shown that the severity of these disorders can be suppressed, or at least relieved, by aspirin. Aspirin is a cornerstone treatment for the prevention of diabetic complications such as cardiovascular diseases. Although aspirin mediates its antithrombotic effects by inhibiting the cyclooxygenase (COX 1) enzyme, it not only effects platelet function, but also RBCs. Aspirin decreases the platelet reactivity amplified by RBCs through the down-regulation of the P-selectin and integrin IIb/IIIa, which in turn reduces the pro-thrombotic phenotype of RBCs [17,18]. Moreover, the interaction between platelets and RBCs in blood vessels, during thrombus formation, is strongly influenced by any reduced deformability of RBC membranes. This rheological mechanism can be reduced by aspirin, which decreases the rate of RBC aggregation [19,20]. Furthermore, evidence suggests that aspirin induces RBCs' nitric oxide synthase, which is well known to be helpful in regulating of the vascular tone and immune response [21]. The efficiency of all these processes seems to be reduced in RBCs by an acetyl-hydrolase activity that rapidly decreases the half-life of aspirin in blood [22–24]. Despite the significant number of studies that have attempted to understand the separated roles of non-enzymatic glycation and aspirin acetylation on the biological processes of RBCs, the exact mutual and synchronous interaction between these two modifications has so far been poorly investigated.

In this study, we carried out the first analyses of the simultaneous impact of protein glycation and aspirin acetylation on RBC proteins, with a special emphasis on haemoglobin. An experimental mass spectrometry (MS) approach, previously applied to serum albumin [25] and human plasma [26], was used to obtain qualitative information, by detecting acetylation and glycation sites, as well as quantitative reports determining the levels of these modifications.

2. Materials and methods

2.1. Chemicals

EDTA (10.8 mg) – Vacutainer tubes were from BD Vacutainer®. Dulbecco's phosphate-buffered saline (DPBS; 1X, pH 7.4) was from Invitrogen™. NaCl 0.9% was from Bichsel. Mouse anti-human CD235a, Glycophorin A/RPE antibody was from Dako. Aspirin was from Aspegic Inject®. EDTA-free protease inhibitor cocktail (PIC) tablets were from Roche. Anti-human Nε-acetyl-lysine monoclonal antibody was from Cell Signalling Technologies®. ECL™ detection reagent was from GE Healthcare. Protein assay dye reagent concentrate (liquid) was from Bio-Rad. D-glucose [$^{12}C_6$] (lyophilized powder, ≥99.5%), Naphtol Blue Black (lyophilized powder, dye content *ca* 80%), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP; 0.5 M, pH 7.0), iodoacetamide (IAA, crystalline, ≥99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilized powder, 500 U), HPLC-grade water (CHROMASOLV®) and HPLC grade acetonitrile (ACN; CHROMASOLV®, ≥99.9%) were purchased from Sigma–Aldrich®. Bovine serum albumin (BSA, lyophilized powder, ≥96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, ≥99.0%), acetic acid (99.5%), and formic acid (FA; 98.0%) were from Fluka.

2.2. RBC preparation and lysis

RBC fraction was obtained by centrifuging whole blood from three blood bank donors. Briefly, EDTA-Vacutainer tubes containing blood were centrifuged at 900 rpm, at 37 °C, to remove the platelet rich plasma fraction. Then, the remaining part was centrifuged at 3500 rpm for 10 min to separate and remove the plasma from the RBCs. Carefully 1 mL of erythrocytes was aspirated from the bottom of the tube and transferred to a Falcon tube. RBCs were washed four times with 0.9% NaCl and centrifuged at 3690 rpm for 10 min to minimize the presence of contaminants. Before the final washing step, 100 μL of cell suspension was used to determine the concentration RBCs using a Sysmex KX21 N haematology analyser (Sysmex Corporation). A sample concentration of 2.51×10^6 cells/μL was diluted down to 10^4 cells/μL and incubated with an anti-human antibody against CD235a (Glycophorin A) for subsequent fluorescence-activated cell sorting (FACS) analysis. The level of cell purity was assessed using an Accuri C6 fluorescence flow cytometer (BD Accuri™). After sedimentation, the supernatant was removed, and a cell pellet (~1 mL) was lysed by adding eight volumes of deionized water. Cell debris was separated by centrifugation at 13,000 rpm for 30 min at 4 °C, and protease inhibitors were added to the RBC extract at a dilution of 1:7 before aliquoting. The three RBC protein extracts were then pooled together.

2.3. RBCs incubation with aspirin and glucose

Four aliquots of 225 μL of RBC protein extract were diluted in phosphate buffer to reach a final volume of 400 μL, and were subsequently incubated with (1) 500 μM aspirin for 30 min at 37 °C; (2) 30 mM glucose for 24 h at 37 °C; and (3) sequentially, with 30 mM glucose at 37 °C for 24 h followed by 500 μM aspirin for 30 min at 37 °C. As negative control, a further aliquot was incubated using the same time periods, but without glucose and aspirin. Additional five conditions were created in which 100 μL of RBC extract were incubated for 24 h with increasing glucose concentrations (0, 10, 30, 50 and 100 mM) followed by 30 min of aspirin exposure at 37 °C. As a positive control,

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