Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Impact of high glucose concentration on aspirin-induced acetylation of human serum albumin: An *in vitro* study

Francesco Finamore[a], *Feliciano Priego-Capote*[b], *Florent Gluck*[a], *Anne Zufferey*[a], *Pierre Fontana*[c], *Jean-Charles Sanchez*[a,*]

[a] *Translational Biomarker Group (TBG), Department of Human Protein Sciences, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland*

[b] *Department of Analytical Chemistry, University of Còrdoba, Annex C-3 Building, Campus of Rabanales, Spain*

[c] *Division of Angiology and Haemostasis, Geneva Platelet Group (GPG), Geneva University Hospital, Geneva, Switzerland*

ARTICLE INFO

Article history:
Received 17 July 2013
Received in revised form
19 November 2013
Accepted 4 February 2014
Available online 5 March 2014

Keywords:
Aspirin
Acetylation
Glycation
Human serum albumin
Lysine
Mass spectrometry

ABSTRACT

Aspirin (ASA) plays a key role in protecting high risk cardiovascular patients from ischaemic events. The modifications underlying its effects are the results of the trans-acetylation that occurs between ASA and the amino groups made up of lysine and N-terminal residues. ASA's effects have also been demonstrated on several plasma proteins, including human serum albumin (HSA). However, its beneficial effects seem to be lower in diabetic patients, suggesting that protein glycation may impair ASA's acetylation process. Using immunoblotting and mass spectrometry, this study characterized the degree of HSA acetylation mediated by ASA *in vitro*, as well as the impact of high glucose concentrations. Glycation's influence on HSA acetylation might impair the latter's biological functions, leading to a potential failure of ASA to prevent cardiovascular complications in diabetes.

1. Introduction

Since its serendipitous discovery and thanks to mid-20th century improvements, aspirin, also known as acetylsalicylic acid (ASA), has become a landmark drug in the history of medicine. ASA is the world's most well-known non-steroidal anti-inflammatory drug; this is due to its pleiotropic effects, either in the ordinary treatment of pain, fever and inflammation, or in clinical practice [1,2]. Indeed, it is widely used as a preventive agent in atherothrombotic diseases, because of its inhibition of the COX-1 platelet enzyme [3]. It also enhances the resolution of inflammatory states by increasing the activity of the COX-2 vascular enzyme [4], and thus prevents the risk of cardiovascular ischaemic events [5,6]. ASA plays a relevant role in lowering insulin resistance, by reducing levels of pro-inflammatory cytokines [7], and furthermore it exerts its therapeutic action through the transfer of its acetyl moiety to the primary amino groups of proteins located at lysine and N-terminal residues. Under

Abbreviations: ASA, acetyl salicylic acid; HCD, high energy collisional dissociation; HSA, human serum albumin; MS, mass spectrometry.

* *Corresponding author at:* Translational Biomarker Group, Department of Human Protein Sciences, University of Geneva, Rue Michel Servet 1, 1211 Geneva 4, Switzerland. Tel.: +41 022 3795486; fax: +41 022 3795505.
E-mail address: Jean-Charles.Sanchez@unige.ch (J.-C. Sanchez).

http://dx.doi.org/10.1016/j.euprot.2014.02.001

physiological conditions (37 °C, pH 7.4) this reaction is fast and follows a first order kinetic dependent only on the concentration of ASA. After usual therapeutic doses, normal ASA plasma levels range from 0.7 to 2.2 mM, however toxicity can occur when serum concentrations exceed 2.9–3.6 mM and can become fatal beyond 5.4 mM [8]. Several studies have shown the effect of ASA-induced acetylation on different plasma proteins. It increases haemoglobin's affinity to oxygen and consequently reduces the severity of sickle-cell disease [9,10]. Acetylation of fibrinogen by ASA improves the porosity of the fibrin cloth making it more accessible to plasmin for the fibrinolytic process [11]. ASA also acts on low density lipoproteins, reducing their oxidation level and thus exerting a preventive effect against atherosclerosis [12]. ASA-mediated acetylation has also been detected on human serum albumin (HSA) [13]. HSA is known to be the most abundant circulating plasma protein, and because it has multifunctional properties it plays key roles in metabolism. It is the main transporter of drugs, hormones, fatty acids and other small metabolites. It regulates oncotic pressure, prevents oxidative damage and has an intrinsic esterase activity. Previous studies showed the involvement of different amino acidic residues in HSA's reactions with low ASA concentrations (0.1–0.5 mM) [14,15]. It was demonstrated that the acetylation of some of these sites had an impact on HSA's pseudo-esterase catalytic activity, and on its affinity for binding to anionic drugs and metabolites such as bilirubin and prostaglandins. However, HSA's beneficial effects are lower in poorly monitored diabetic patients, *via* a process termed as 'aspirin resistance'. This can be attributed to insufficient doses of ASA, drug interactions, or the genetic polymorphism of genes involved in thromboxane biosynthesis and turnover [16,17]. In addition, because high plasma glucose levels encourage protein glycation, they might have a significant effect on aspirin resistance and other metabolic disorders. Glycation is a non-enzymatic post-translational modification, which is boosted under hyperglycaemic conditions (blood glucose concentration $\geq$ 11 mM). It occurs in a condensation reaction between the carbonyl group in glucose (or any other reducing sugar, such as fructose and derivatives) and a protein's free amino residues (mostly N-terminal and lysine). This process leads to the formation of Amadori compounds which then undergo a series of oxidation, dehydration and fragmentation reactions, finally generating advanced glycation end-products (AGE) [18,19]. AGEs are associated to long term diabetic complications, such as retinopathy [20,21], nephropathy [22–24] and macroangiopathy [25,26]; this is due to the formation of cross-linked structures [27,28] that alter the structure/function of proteins, their turn-over, and tend to accumulate in specific tissues, impairing their homeostasis [29,30].

Apart from its clinical relevance as a more rapid indicator for glycaemic monitoringl than glycated haemoglobin (HbA1c), glycated HSA has also been described as having altered functions in diabetes (glycated albumin levels in plasma are considered to indicate the glycaemic state over the last 3 weeks, while HbA1c indicates the glycaemic state over the last 3 months) [31–33]. Indeed, it was shown that glycation significantly impairs HSA's antioxidant properties [34] and its drug-binding capacity [35]. Furthermore, the alterations to HSA's biophysical properties, induced by glycation, increase its affinity to the receptor for advanced glycation end-products (RAGE) [36]. This leads to several cellular dysfunctions, such as: the intracellular accumulation of lipids in macrophages [37]; the increase of their inflammatory activity by inducing NF-κB [38]; the secretion of IL-6 by adipocytes [34]; and the promotion of neuronal apoptosis in the human brain [39], contributing to the development of Alzheimer's-like diseases.

The potential protective role of ASA in inhibiting the glycation mechanism was previously shown in lens crystallins [40,41], collagen [42], haemoglobin [43] and, most recently, fibrinogen [44]. However, the impact of increased blood glucose concentrations and glycation on ASA-mediated acetylation has been poorly investigated. To date, only Watala et al. have shown the impairment of ASA-induced protein acetylation on platelet membrane proteins in diabetes mellitus, due to an increased level of glycation [45]. Further research is required to determine how high glucose concentrations might hamper the protective action of ASA at the protein level. This study aimed to highlight HSA's acetylation response after incubation, from low to very high ASA concentrations, but also to elucidate which amino acidic residues showed the highest reactivity towards ASA. The study's second aim was to examine the competition between glycation and acetylation in order to demonstrate how these two reactions take place at the protein level. Finally, the characterization of the preferential sites where these two modifications occur was performed using mass spectrometry (MS), and the quantification of glycated and acetylated peptides of HSA was carried out using a label-free approach. This proof-of-concept study lays the foundations for a better understanding of the interaction between the ASA-mediated acetylation and glycation processes on proteins, and gives new insights into their potential antagonist effects in the pathophysiology of diabetes.

2. Materials and methods

2.1. Chemicals

Dulbecco's phosphate-buffered saline (DPBS; 1×, pH 7.4) was from Invitrogen™. Human serum albumin (HSA, solution 20%) was obtained from CSL Behring AG. Aspirin (ASA, acetylsalicylic acid) was purchased from Aspegic Inject®. Anti human Nε-acetyl-lysine monoclonal antibody was from Cell Signaling 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, $\geq$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, $\geq$99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilized powder, 500 U), HPLC-grade water (CHROMASOLV®) and HPLC grade acetonitrile (ACN; CHROMASOLV®, $\geq$99.9%) were purchased from Sigma–Aldrich®. Bovine serum albumin (BSA, lyophilized powder, $\geq$96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, $\geq$99.0%), acetic acid (99.5%), and formic acid (FA; 98.0%) were from Fluka.