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A high glucose level is associated with decreased aspirin-mediated acetylation of platelet cyclooxygenase (COX)-1 at serine 529: a pilot study.

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Short Title: Impact of aspirin-induced acetylation on platelet COX-1 in diabetes

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Abstract

Diabetes is a major risk factor for cardiovascular diseases. Although aspirin is considered a cornerstone of the prevention and treatment of atherothrombotic-related ischemic events, this antiplatelet drug appears to be less effective in patients with poorly controlled diabetes. It has been suggested that the glycation of platelet proteins plays a pivotal role in poor responsiveness to aspirin. However, a direct effect on the critical residue (serine 529, or Ser 529) of the catalytic pocket of cyclooxygenase 1 (COX-1) has never been demonstrated. This pilot study aimed to elucidate the impact of hyperglycaemia on aspirin acetylation of COX-1 using a targeted mass spectrometry approach. We observed that high glucose concentration had a direct impact on the level of acetylation of the COX-1 Ser 529 residue, whereas it's overall acetylation level remained unchanged. Moreover, the functional aspirin-induced inhibition of COX-1 was dose-dependently impaired as glucose concentrations increased. These *in vitro* findings were in line with data obtained using platelets from diabetic patients. These data provide new insights into the interplay between glucose and aspirin on platelet proteins and their effects on platelet COX-1. They also suggest a potential mechanistic explanation for the phenomenon of poor response to aspirin in diabetic patients.

Introduction

Cardiovascular diseases are the main causes of morbidity and mortality in patients with diabetes mellitus [1, 2]. Antiplatelet therapy [3, 4] was long thought to protect these patients, either as primary or secondary prevention [5-8]. However, platelets from diabetic patients are characterised by an enhanced reactivity phenotype and a decreased biological response to aspirin [9, 10]. This is in line with results showing no clear benefits of aspirin as a primary prevention for diabetic patients [5, 11] and a consistent absence of increased bleeding-risk related to that drug [12].

Aspirin's antithrombotic effect is mediated by the inhibition of cyclooxygenase (COX) -1 in platelets. This hampers the conversion of arachidonic acid to prostaglandin G_2/H_2 and finally to thromboxane (Tx) A_2 [13, 14]. The exact site where aspirin exerts its function on COX-1 was revealed about twenty-five years ago, using ovine COX-1, which has a 95% sequence similarity with its human counterpart [15, 16]. According to structural characterisations, COX-1 inhibition by aspirin is mainly due to the irreversible acetylation of the serine residue 529 (Ser 529) located, together with Tyr 385, at the apex of the peroxidase catalytic site, at the bottom of a long hydrophobic channel. This process occurs through a two-step mechanism involving: 1) the electrostatic interaction of the salicylate group of aspirin with the guanidine moiety of the Arg 120 side chain, located at the entrance of the channel; and 2) the position of the aspirin acetyl group, in proximity to the hydroxyl groups of Ser 529, through hydrogen bonds and van der Waals interactions with Tyr 385 [17, 18] (Figure 1). The resulting acetylation of Ser 529 prevents arachidonic acid from having access to its binding site on the enzyme [19], leading to the inhibition of TxA_2 synthesis. The extent of TxA_2 inhibition was shown to be inversely correlated with the level of COX-1 acetylation, highlighting the importance of the acetylated Ser 529 as a specific indicator of drug action [20].

To date, a large body of evidence supports the relationship between the less-than-expected response to aspirin and diabetes [9] as a result of residual platelet hyper-reactivity [10] due

78 to accelerated platelet turnover [21-24] and increased oxidative stress [25]. Besides the
79 relevance of extra-platelet sources of TxA_2, such as monocyte/macrophage COX-2 [26,
80 27] and increased levels of F_2-isoprostanes [25, 28], the residual platelet activity may also
81 result from the impairment of COX-1 aspirin acetylation due to hyperglycaemia favouring
82 non-enzymatic glycation. The mutual influence of glycation and acetylation has been
83 reported previously on platelets from diabetic patients [29], suggesting that the poor
84 response to aspirin could be explained by an extensive glycation of platelet proteins that in
85 turn interferes with their state of acetylation. Furthermore, a number of glycation and
86 acetylation sites were detected on purified ovine COX-1, suggesting that very high glucose
87 concentrations might affect aspirin's ability to acetylate the enzyme [30]. However, the
88 potential impact of high glucose levels on the aspirin-induced acetylation of the critical
89 residue (Ser 529) of human COX-1 remains unknown. Using a targeted mass spectrometry
90 method capable of parallel reaction monitoring (PRM), we showed that aspirin's
91 acetylation of platelet COX-1 Ser 529 was markedly lower in presence of *in vitro* high
92 glucose concentrations. Furthermore, high glucose levels dose-dependently decreased the
93 aspirin-induced inhibition of COX-1 activity. These findings were in line with data
94 obtained with platelets of a representative cohort of patients with poorly controlled
95 diabetes.

96 **Material and Methods**

97 **Patient and healthy control selection and blood sampling.** Diabetic patients (n = 3) were
98 recruited in the diabetic outpatient clinic of Geneva University Hospitals. The inclusion
99 criterion was a level of glycated haemoglobin (HbA1c) ≥ 8%. The exclusion criteria were
100 regular insulin treatment and aspirin or an intake of non-steroidal anti-inflammatory drugs
101 during the 60 days prior to blood collection, in order to avoid any drug-induced protein
102 acetylation. Age and sex-matched healthy control subjects (n = 3) with no intake of
103 medication during the last 60 days and with HbA1c < 6% were also recruited at Geneva
104 University Hospitals. All study participants gave their written informed consent, and the

Central Ethics Committee of Geneva University Hospitals approved the study protocol (CER 12-019). Blood was drawn from an antecubital vein into 6 mL Vacutainer tubes containing 10.8 mg EDTA (BD Vacutainer®) (PMID:26883867).

Platelet sample preparation. Blood was centrifuged at 150 g for 15 min at 37°C, and the platelet-rich plasma (PRP) was used for an additional centrifugation step to remove residual erythrocytes and leukocytes [31] (PMID:26883867). The homogeneity of platelet population was evaluated by FACS analysis using the Accuri C6 fluorescence flow cytometer (BD Accuri™). Briefly, 10^6 platelets were incubated with 20 µL FITC labelled anti-human CD42b (BD Pharmigen™), 2 µL PC5 labelled anti-human CD45 (IO Test®) and 1 µL RPE-labelled anti-human CD 235a for 15 min at room temperature and in dark conditions. The level of contamination of erythrocytes and leukocytes from the PRP sample was measured at around 0.3% for both cell populations. PRP was then centrifuged at 1000 g for 10 min in the presence of 25 µM PGI_2; then platelet pellets were washed with Dulbecco's phosphate-buffered saline (DPBS; 1X, pH 7.4, Invitrogen™). Platelets were then lysed in 0.2% Rapigest (Waters, Milford, USA) DPBS buffer (pH 7.4) and sonicated five cycles of 20 sec with breaks on ice (40 sec). Samples were then centrifuged at 16000 x g for 20 min at 4°C. Protein concentration was measured by Bradford assay (BioRad, Hercules, CA, USA).

Glucose and aspirin *in vitro* incubation and COX-1 functional assay. Protein extracts from the different platelet fractions were used in two different experimental protocols aiming to characterise the influence of glucose on aspirin-mediated acetylation. The first protocol addressed how exposing the platelet proteins of healthy subjects to a high glucose concentration affected the acetylation process induced by aspirin in order to mimic an acute hyperglycaemic environment. For this purpose, 50 µL of platelet protein sample from each control subject were pooled together and used across four test conditions in which 270 µg of proteins were incubated either with 1) 500 µM of aspirin for 30 min at 37ºC, or 2) 30 mM glucose for 24 hours at 37ºC, or 3) 30 mM glucose followed by 500 µM of aspirin.

Condition 4) used the last 270 µg sample, incubated at 37ºC for the same period without glucose or aspirin (negative control). Samples were then immediately processed for protein digestion. The second experimental protocol looked at the comparison between chronic hyperglycaemic samples (from diabetic patients with HbA1c ≥ 8%) to control samples (from the previous healthy subjects with HbA1c < 6%) in their response on acetylation processes after *in vitro* exposure of 30 µL of platelet proteins with 500 µM aspirin for 30 min at 37ºC.

Platelet COX-1's activity in the presence of glucose and aspirin was monitored using a COX Activity Assay Kit (Cayman Chemical, Ann Arbor, MI) that measures calorimetrically COX-1's peroxidase reaction. First, platelet protein extracts were incubated with 500 µM aspirin for 30 min, 30 mM glucose for 24 hours and with a sequential incubation with 30 mM glucose for 24 hours followed by 500 µM aspirin for 30 min. A platelet protein sample containing neither glucose nor aspirin was used as a reference, while sample incubated with a selective COX-1 inhibitor (SC-560) was used as negative control. For the end-point study, platelet proteins were incubated with an increasing glucose concentration (0, 10, 30, 50, 100 mM) for 24 hours and subsequently exposed with 500 µM aspirin for 30 min. The same conditions without added aspirin were used as a reference. COX-1's activity was determined by an absorbance reading taken at 590 nm. Samples were analysed in triplicate.

Protein digestion. After incubation, samples from both studies were ultra-filtrated with Amicon Ultra-0.5 mL, 3K cut-off filters (Millipore™) in order to remove excess salts, aspirin and glucose and to exchange the solubilisation buffer with 50 mM ammonium bicarbonate (Sigma-Aldrich®), pH 8.0. The protein amount was estimated again using a Bradford assay, and 100 µg of proteins were used for the subsequent trypsin digestion. Cysteine residues were reduced using 5 mM TCEP for 30 min at 60ºC and the resulting free sulfhydryl groups were alkylated with 20 mM iodacetamide for 30 min at room temperature in dark conditions. Then, a solution of 200 ng/µL of trypsin was freshly

prepared and 10 µL (corresponding to a ratio of 1:50 w/w) was added to each protein sample and incubated for 16 hours at 37ºC. Afterward, 0.5% trifluoroacetic acid was added to each protein digest and incubated for 40 min at 37ºC under shaking in order to induce the acidic cleavage of the Rapigest surfactant. After centrifugation at 16000 g, supernatants were lyophilised and then reconstituted in 5% acetonitrile / 0.1% formic acid in order to be subsequently desalted and concentrated using a C18 MacroSpin Column™ (Harvard Apparatus). Peptides were eluted with 50% acetonitrile / 0.1% formic acid and then evaporated to dryness before the final reconstitution in 5% acetonitrile / 0.1% formic acid for the subsequent mass spectrometry analysis. Samples were analysed in triplicate using a targeted PRM mass spectrometry approach.

Detection and quantitation of platelet COX-1 acetylation using PRM. PRM analysis was carried out using a Q Exactive Plus (ThermoFisher, San Jose, CA) mass spectrometer, equipped with a Thermo EASY-nLC and coupled with an EASY-Spray source operating at 1.8 kV in positive ion mode. Peptides were trapped on a 2 cm x 75 µm i.d., PepMap C_{18} precolumn packed with 3 µm particles of 100 Å pore size. Subsequent separation was achieved in a 50 cm x 75 µm i.d., PepMap C_{18} column packed with 2 µm particles of 100 Å pore size heated at 50°C. Liquid chromatography was performed using a 60-min gradient at a flow rate of 250 nL/min, with 0.1% (v/v) formic acid (Fluka) in HPLC-grade water (CHROMASOLV®) as mobile phase A and 0.1% (v/v) formic acid in HPLC-grade acetonitrile (CHROMASOLV®) as mobile phase B. The gradient programme was as follows: 5% B for 5 min; ramping up to 35% B over 55 min; rapid ramping up to 90% B over 10 min and and then the column was washed for 15 min. The column was re-equilibrated to 5% B for 24 min after each run. Sixteen target masses (doubly and triply charged ions) corresponding to 8 COX-1 peptides in their acetylated and non-acetylated states (Supplemental Table 1), had been chosen previously based on their stability, sequence (modified and unmodified) and strong transition signals. This inclusion list triggered targeted scans at a resolving power of 17,500, with an isolation width of 3 Th

around the m/z of interest, an AGC target of 2 x 10^5, a maximum injection time of 100 ms and a normalised collision energy of 27% in a higher-energy c-trap dissociation (HCD) cell. According to these parameters, an entire cycle time of ≈ 2 sec was required to acquire all transitions, with an average of 12 points per peak (30–40 sec elution width). Data were analysed using the targeted MS/MS feature implemented in Skyline v3.5 software [32]. In order to confirm the identity of the peptides, a spectral library of annotated reference MS/MS spectra was created from a platelet proteins dataset and acetylated COX-1 previously generated using a classic DDA approach. Peptides are quantified by extracting the peak areas of accurate fragment ions (< 6 ppm) and integrated across the peptides' elution profile. For each peptide, transition peak areas for the most intense fragment ions containing the modification site were normalised on the total ion current (TIC) from the MS2 scans for each run. Normalized intensities of the selected fragments were summed together for peptide quantification.

Identification of the preferential glycation sites on COX-1. Human recombinant COX-1 (BPS Bioscience Inc.) was incubated with 1 M glucose for 24 hours in order to speed-up the kinetic of the glycation process. Glycated COX-1 was then separated by SDS-PAGE and in-gel digested with trypsin (1:50 w/w). Peptide extraction from gel slices was performed using 30% acetonitrile / 0.1% trifluoroacetic acid, followed by evaporation to dryness and reconstitution in 200 mM NH_4Ac (ammonium acetate), 50 mM $MgCl_2$, pH 8.1, for glycated peptide enrichment. Fractionation of glycated peptides of human COX-1 was carried out via boronate affinity chromatography, as previously described [33]. The peptide glycated fraction was collected and subjected to a data-dependent mass spectrometry method on a Q Exactive Plus coupled with HCD fragmentation (normalised collision energy 27%). MS survey scans were acquired at a resolving power of 70,000 in profile mode with a 200–1600 mass range. MS/MS data were acquired at a resolving power of 17,500 in centroid mode with a dynamic exclusion of 30 seconds and an isolation window of 1.6 m/z. Peak list data were queried against the human UniProtKB/Swiss-Prot database (20,197 sequences) with an error tolerance

of 10 and 6 ppm for precursor and fragment ions, respectively. Alkylation of cysteine residues was selected as the fixed modification, whereas oxidation of methionine and glycation of lysine, arginine, serine, threonine, tyrosine and N-terminal residues were selected as variable modifications. The confidence of peptide identification was validated by the exact assignment of the glycation motif on the peptide sequence.

***In silico* structure prediction and molecular docking analysis of human COX-1.** The prediction of the three-dimensional (3D) structural model of human COX-1 was performed using the Phyre2 (v 2.0) homology prediction tool [34]. Briefly, the primary sequence of human COX-1 is queried in PSI-BLAST to detect sequence homologues and a position-specific scoring matrix (PSSM) is calculated from the multiple alignments. Then, the secondary structure's elements are estimated using the Psi-pred neural-network prediction tool [35]. A hidden Markov model (HMM) is generated from the query sequence and matched against a library of HMMs from proteins of known structure. The 3D model of human COX-1 is then constructed depending on this alignment, that in turn depends on the similarity of residue probability in each position and on the secondary structure. A confidence higher than 90% and an identity (accuracy of the model) higher than 40% establish a true structural homology between the protein and the template. The model was built up using the known structure PDB:c2oyuP as a template that yields a sequence coverage of 92% with the highest confidence value of 100%, and an identity of 94% after alignment. The structure generated was then visualised using PyMOL (The PyMOL Molecular Graphic System, Version 1.7 Schrödinger, LLC) in order to understand the spatial distribution of the preferential sites that were found to be acetylated by aspirin. To predict the potential binding of glucose in the COX-1 catalytic pocket, a molecular docking simulation was performed using AutoDock Vina [36], the homology model of human COX-1 as the receptor and the linear form of glucose as the ligand. A grid box size of 12 x 12 x 12 with a grid spacing of 1 Å was centred on the entrance of the pocket. A total of 20 poses were calculated and a "score" (binding energy, kcal/mol) was determined for each

ligand conformation via a scoring function, which uses the AMBER force field to infer the free energy associated with the complex.

Statistical analysis. The experimental data were analysed for statistical significance using R software (version 3.2.1) [37]. A Kolmogorov-Smirnov test was used to assess the normality of data distributions. A Student's two tailed *t*-test was used to compare the acetylation level of COX-1 sites in each group pair, across the 4 *in vitro* condition tests. A one-way analysis of variance (ANOVA) was used to address the level of significance of acetylated COX-1 sites in healthy subjects and patients with poorly controlled diabetes, considering the two nominal variables of the data set (biological and technical). A value of $p < 0.05$ was considered a significant difference between the sample conditions.

Results

Impact of high glucose on aspirin-induced COX-1 acetylation

The effect of high glucose concentration on aspirin-mediated acetylation of human COX-1 was assessed using a PRM mass spectrometry approach on a pool of platelet protein extracts from healthy subjects (HbA1c < 6) treated *in vitro* first with 30 mM glucose for 24 hours followed by 500 µM aspirin incubation for 30 minutes. The choice to use such a targeted method relied on the fact that classical shotgun proteomics approach was not sensitive enough to detect the acetylated form of COX-1 Ser 529 residue, despite several other platelet proteins, target of aspirin-mediated acetylation, were unambiguously identified (refer to Data in Brief for details). Samples were first incubated with glucose before aspirin exposure. We detected and quantified eight peptides/sites in the acetylated and non-acetylated states of COX-1, with a correlation coefficient (r^2) of 0.996 between triplicates (Supplemental Table 2). We found that a concentration of glucose as high as 30 mM had no significant impact on the aspirin-induced acetylation of most COX-1 sites (Supplemental Figure 1). The acetylation of Ser 529 residue was quantified using the seven most abundant specific fragment ions containing the acetyl group (Figure 2 A). This

analysis showed a significant decrease in aspirin-induced acetylation after incubation with glucose ($p = 0.015$, fold change = 4.3, Figure 2 B-C) compared to aspirin incubation only. These results suggest that high glucose level influences the acetylation extent of COX-1 Ser 529 residue by aspirin, leaving the other quantified sites mostly unaffected (with the only exception of Lys 168, fold change = 1.3). A general accepted hypothesis rely on the fact that non-enzymatic glycation might be directly involved in the poor response of COX-1 aspirin-acetylation, as described above. We attempted to verify this hypothesis using a very high glucose concentration (1M) in order to favour as much as possible the COX-1 glycation response. Qualitative data on glycated COX-1 revealed the presence of 11 preferential glycation sites (Supplemental Table 3, Supplemental Figure 2), of which 7 were also shown to be targets of aspirin-induced acetylation (Lys 156, Lys 167, Lys 168, Lys 221, Lys 252, Lys 316, Lys 472). Glycation on Ser 529 or on any other residue belonging to the catalytic pocket of COX-1, was not detected with the experimental conditions used in this experiment.

Functional impact of glucose on *in vitro* aspirin-induced inhibition of COX-1 enzymatic activity

To determine whether Ser 529's reduced level of acetylation was associated with glucose impairing aspirin's inhibition of COX-1 activity, we evaluated the conversion of arachidonic acid into PGH_2, under different conditions, using a pooled sample of platelet proteins from healthy subjects. As expected, 500 µM aspirin alone inhibited COX-1's activity by 90% while glucose alone showed no effect. When aspirin was added after glucose pre-incubation, we observed a significant decrease in the inhibition of COX-1 activity (Figure 3 A). We extended these findings with different glucose concentrations that showed a dose-dependent effect on aspirin-induced inhibition of COX-1 activity. Despite aspirin exposure, COX-1 activity increased as glucose concentrations increased, whereas high glucose concentrations alone had no effect on enzyme activity (Figure 3 B).

These results indicate that high glucose levels hamper the normal aspirin function, leading to a partial and progressive decrease of COX-1 inhibition.

Ser529 of Cox1 is differentially acetylated by aspirin in 3 diabetic patients compared to 3 matched controls

The previous data are obtained from an *in vitro* approach that mimics an acute gluco-toxicity stress induced by high glucose level incubation on platelet proteins of healthy subjects in a relatively short lapse of time. However, this situation does not properly reflect the long-lasting effects of glucose, typical of diabetes, on the aspirin-mediated acetylation process. To validate the results obtained *in vitro*, chronic hyperglycaemia's effect on aspirin-mediated acetylation was assessed on the platelet protein extracts of patients with poorly controlled diabetes (HbA1c > 8%) in comparison to healthy controls (HbA1c < 6%) after *ex vivo* exposure to 500 µM aspirin for 30 min. The mass spectrometry method used to quantify COX-1 peptides yielded average coefficients of variation (CV) of 22.3% and 26.4% for the control (n = 3) and diabetic (n = 3) groups, respectively. An intra-class correlation of $r^2 = 0.99$ was calculated to assess the technical variability between runs (Supplemental Table 4). A total of six COX-1 acetylated peptides (and their non-acetylated counterparts) were detected and quantified, showing no evident differences in the acetylation levels of 3 out of 6 COX-1 sites between the two groups. In particular, the acetylation on Lys 221, Lys 252 and Lys 472 were shown to be not significantly affected in diabetic patients when compared to controls, whereas Lys 167 and Lys 168 showed slight differences between the two groups, with a fold-change of 1.26 and 1.29 ($p = 0.04$ and $p = 0.02$, respectively), suggesting that diabetes did not influence drastically the aspirin-acetylation on these residues (Supplemental Figure 3). In contrast, aspirin-induced acetylation of Ser 529 of COX-1 was significantly lower in diabetic patients compared to controls with a respective fold change of 2.7 before and after aspirin exposure ($p < 0.001$, Figure 4 A), as detailled by the extracted ion chromatograms (XICs) in each group (Figure 4 B). These data strongly support the results obtained after *in vitro* incubation of platelet proteins from healthy subjects with high glucose concentration described above.

In silico 3D structural study of the interplay between glucose and aspirin-induced Ser 529 acetylation

Peptide sequence information from MS data allowed to map the exact location of acetyl modification on COX-1 protein structure and to calculate the likely role of glucose on the lower acetylation effect of aspirin at Ser 529 residue. To overcome the absence of any available 3D structures of human COX-1, we used an *in silico* model of the protein as a template: the Protein Data Bank's c2oyuP structure. We observed that all the acetylated residues (except Ser 529) found in our study lie on the protein's solvent accessible surface (Figure 5 A-C), whereas Ser 529 is situated in a buried region of the enzyme surrounded by a 7 Å-width hydrophobic channel (Figure 5 D), corresponding to the peroxidase and cyclooxygenase domains of COX-1. Additional acetylated residues were not detected in the enzyme's catalytic site. A docking analysis was performed to gain insight on how glucose can influence the acetylation of Ser 529. Two major clusters of glucose binding poses were located close to the Arg 120 and Tyr 354 residues surrounding the active site groove's entrance, with free energy values ranging from -3.4 and -4.3 kcal/mol (Supplemental Figure 4 A-B). The glucose conformations with the lowest binding energies were chosen from both clusters and are represented in Figure 6. Glucose can be positioned at the exterior of the pocket, where it interacts with the Arg 120 residue through hydrogen bonds between the carbonyl oxygen of the sugar and the η amines of the guanidine group, together with those formed between the hydroxyl group of carbon atoms 5 or 6 of the sugar and the ε amine of the guanidine group (Figure 6 A). Alternatively, glucose can be stabilised in the pocket, at the interface between the Arg 120 and Tyr 354 residues, by two potential sets of H–bonds: 1) between the hydroxyl group of carbon atoms 4 or 5 and the η amines of the guanidine group of the Arg 120 residue, or 2) between the hydroxyl group of carbon atom 3 and that of the aromatic moiety of the Tyr 354 residue (Figure 6 B). According to this *in silico* model, irrespective of the two conformational options detailed above, glucose is positioned in proximity to the cavity that surrounds the COX-1 catalytic

346 domain. In turn, this leads to polar contacts with Arg 120, which most likely interfere with
347 aspirin's specific action on this latter residue and, subsequently, prevent aspirin's access
348 to the pocket and from providing its acetylating effect on Ser 529.

349 **Discussion**

350 The present study describes the impact of *in vitro* high glucose concentration and diabetes
351 on aspirin-induced acetylation of platelet-derived COX-1's Ser 529 residue. These results,
352 together with the *in silico* analysis of COX-1's 3D structure unveil mechanistic hypothesis
353 of the less-than-expected response to aspirin. These data expand on those found with ovine
354 COX-1 and *in vitro* glycation [30].
355 High residual platelet reactivity and suboptimal response to antiplatelet agents is a
356 phenotype frequently observed in diabetic patients. Potential explanations rely on 1) an
357 increase in newly produced platelets from bone marrow megakaryocytes with newly
358 replenished cargoes of proteins involved in the platelet activation pathway [24, 38]; 2)
359 enhanced oxidative stress due to reduced synthesis of NO; 3) an increase in lipid
360 hydroperoxide and F_2-isoprostane levels [25]; 4) an incomplete inhibition of COX-1. The
361 latter hypothesis is a matter of concern as it would provide a mechanistic explanation
362 specific to aspirin, as opposed to the other hypotheses, which are not drug-specific. Non-
363 enzymatic glycation is indeed a hypothesis that has often been raised, but to date, the
364 mechanism by which glucose might contribute to COX-1's reduced sensitivity to aspirin
365 has remained elusive. Glycation on purified ovine COX-1 was recently shown to interfere
366 with its state of acetylation and vice-versa, suggesting that mutual indirect competition
367 between these two non-enzymatic modifications might occur [30]. In the present study, we
368 demonstrated that high glucose level is responsible of a lower *in vitro* acetylation of platelet
369 COX-1 after incubation with 30 mM glucose and 500 μM aspirin for 24 hours and 30 min,
370 respectively. Since glycation is a low-kinetic process depending on the amount of non-
371 reducing sugar, we selected a higher glucose concentration than the maximal expectable in
372 diabetes (> 11 mM in blood) in order to favour glucose attachment on protein as much as

possible. Then, we used an aspirin concentration that is within the high range of typical *in vivo* therapeutic levels (30 – 100 mg/L corresponding to 0.16 – 0.55 mM) [39, 40] when evaluated in peripheral plasma. Indeed, platelets are likely to be inhibited in the portal circulation where aspirin concentration is probably higher after ingestion [41] (PMID: 6436696). The duration of the exposure (30 min) closely reflects the half-life of aspirin in plasma. The PRM mass spectrometry method used here was sensitive enough to detect several preferential acetylation sites with a high degree of confidence, including Ser 529, whose acetylation level was shown to be much lower at high *in vitro* glucose concentrations (four times lower) and in diabetic patients after aspirin exposure. To the best of our knowledge, this is the first study to address the issue of the impact of hyperglycaemia on aspirin's effect on human platelet COX-1 and, in particular, on the acetylated state of its Ser 529 residue. The glucose-induced decrease of aspirin-induced acetylation of Ser 529 is further supported by the concentration-dependent glucose inhibition of the enzyme activity. This latter experiment showed that glucose by itself had no direct effect on COX-1 activity, but that it interferes with the platelet response to aspirin. These data strongly support previous findings demonstrating that increasing glucose concentrations proportionally attenuate the effect of aspirin in terms of platelet aggregation as evaluated in platelet-rich plasma [42]. Of note, our findings differ from those obtained by Kassassir *et al.* which showed a reduced activity of COX-1 after *in vitro* glycation with glucose alone [30]. However, major differences between the two studies may explain these apparently discrepant results, including different COX-1 species (ovine vs human), different sample complexity (purified COX-1 protein vs whole platelets) or the incubation conditions (extremely high glucose levels [300 mM] vs common clinical levels [10 mM] in our study). Conversely, our results are in line with those showing that the increased platelet reactivity phenotype is associated with elevated baseline levels of TxA_2 in diabetic patients treated with aspirin [43], as indicated by the reduced COX-1 peroxidase activity that is directly proportional to TxA_2 generation.

The analysis of the 3D structure of human COX-1 highlights the prevalence of the acetylated residues on the solvent-exposed surface of the protein, favouring their modification by glucose and/or aspirin in a process mainly governed by the reaction with the fast binding kinetic. This could explain why most of the acetylated COX-1 sites showed no significant variation in their states of acetylation, even in the presence of high glucose levels or in diabetic patients. Unlike the other acetylated sites, Ser 529 is located at the bottom of a narrow, internal hydrophobic channel. Nevertheless, its acetylation level was drastically affected by high *in vitro* glucose levels, result that was further confirmed in patients with poorly controlled diabetes. Our results suggest that in diabetes, the excess of glucose can interfere with the aspirin-induced acetylation of Ser 529, making the enzyme less responsive to this antiplatelet drug. A likely explanation of this effect came from the molecular docking analysis. This showed that glucose could interact with Arg 120 and Tyr 354 residues through the formation of hydrogen bonds, preventing aspirin from entering the narrow, internal hydrophobic channel and contributing to the reduced acetylation of Ser 529. Conversely, glycation may also affect protein function through the modification of amino acid side chains located in the internal pocket's solvent-exposed surfaces, thus hindering aspirin's access to it. However, all the preferential glycated COX-1 sites found in this study lie on the protein surface, and most of them (7 out of 11 residues) were also shown to be a target of aspirin-mediated acetylation. Despite the very high glucose concentration used for this qualitative analysis, we found no evidence of any glycation on Ser 529 or any other internal pocket residue. Finally, a third possible mechanism relies on glucose-induced allosteric conformational changes of residues spatially distant from COX-1's catalytic sites. Additional structural studies are needed to define precisely which mechanisms are involved.

While the findings of the present research are innovative in terms of deciphering the interplay of glucose and aspirin on COX-1 function, a number of limitations have to be pointed out. First, the low sample size of diabetic patients and control subjects somewhat limit our assessment of the precise magnitude of the biological impact of glucose on

aspirin–induced acetylation. However, the amount of peptides (acetylated and non-acetylated) remained similar across the different conditions tested in this study, suggesting that the variation of acetylation between conditions is related to the sole effect of glucose with a low level of experimental variability leading to statistically significant differences. Second, the effect of aspirin-induced acetylation was evaluated *in vitro* with a single dose. Since aspirin does not undergo any bio-activation process to be biologically active, it is likely that *in vitro* experiments are likely to translate *in vivo* for this unique experimental aspirin concentration but no extrapolation can be postulated for lower or higher aspirin concentration. The third limitation relates to the methodological approach used to quantify acetylated peptides detected by PRM. Spiking heavy peptides in complex samples before mass spectrometry acquisition represents the gold-standard method to quantitatively analyse small amounts of native peptides using targeted approaches. Nevertheless, this method is much more complex when dealing with modified acetylated peptides at serine residues. Two other factors may also affect the use of internal standard peptides in this study: 1) the peptide length, too long for several of the target peptides, makes the placement of the heavy amino acid ambiguous for subsequent detection and quantification purposes; and 2) the presence of methionine residues in most of the selected acetylated peptides, which may cause an excessive oxidation during chemical synthesis and storage, leading to an inaccurate quantification of the acetylated peptides. In addition to these factors, it is also worth taking into account the high costs of this specific heavy modified peptide derivatization and synthesis.

In conclusion, our findings demonstrate the impact of diabetes on the aspirin-induced acetylation of platelet COX-1. We showed that the reduced acetylation level of the catalytic Ser 529 site is associated with an incomplete inhibition of COX-1 activity by aspirin, which may contribute to the residual platelet hyper-reactivity observed in diabetes. The data presented here may lead to the clinical evaluation of alternative antiplatelet drugs for diabetic patients, such as P2Y12 inhibitors, especially for those whose diabetes mellitus is poorly controlled [44, 45].

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Authorship

Contributions: F.F. carried out the study's experimental section (including sample preparation, mass spectrometry experiments, COX-1 activity assays), performed data analysis and wrote the first draft of the manuscript; J.L.R. provided fundamental insights for data analysis and helped with project design and manuscript drafting; S.M. recruited the patients and contributed to data interpretation and manuscript revision; J.C.S. and P.F. were responsible for the overall management and supervision of the present study and contributed to data interpretation and manuscript drafting.

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