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Acetylation and glycation of fibrinogen *in vitro* occur at specific lysine residues in a concentration dependent manner: A mass spectrometric and isotope labeling study

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ABSTRACT

Aspirin may exert part of its antithrombotic effects through platelet-independent mechanisms. Diabetes is a condition in which the beneficial effects of aspirin are less prominent or absent – a phenomenon called "aspirin resistance". We investigated whether acetylation and glycation occur at specific sites in fibrinogen and if competition between glucose and aspirin in binding to fibrinogen occurs. Our hypothesis was that such competition might be one explanation to "aspirin resistance" in diabetes. After incubation of fibrinogen *in vitro* with aspirin (0.8 mM, 24 h) or glucose (100 mM, 5–10 days), we found 12 modified sites with mass spectrometric techniques. Acetylations in the α-chain: αK191, αK208, αK224, αK429, αK457, αK539, αK562, in the β-chain: βK233, and in the γ-chain: γK170 and γK273. Glycations were found at βK133 and γK75, alternatively γK85. Notably, the lysine 539 is a site involved in FXIII-mediated cross-linking of fibrin. With isotope labeling *in vitro*, using [^{14}C-acetyl]salicylic acid and [^{14}C]glucose, a labeling of 0.013–0.084 and 0.12–0.5 mol of acetylated and glycated adduct/mol fibrinogen, respectively, was found for clinically (12.9–100 μM aspirin) and physiologically (2–8 mM glucose) relevant plasma concentrations. No competition between acetylation and glycation could be demonstrated. Thus, fibrinogen is acetylated at several lysine residues, some of which are involved in the cross-linking of fibrinogen. This may mechanistically explain why aspirin facilitates fibrin degradation. We find no support for the idea that glycation of fibrin(ogen) interferes with acetylation of fibrinogen.

1. Introduction

Aspirin (acetylsalicylic acid; ASA) prevents atherothrombotic complications largely through acetylation of a serine residue in the platelet COX-1 enzyme [1]. However, aspirin may also acetylate haemoglobin [2] and several plasma proteins including fibrinogen [3–8], a key protein in hemostasis. This effect may be another mechanism through which aspirin reduces the development of atherothrombotic complications. The beneficial effects of aspirin seem, however, to be smaller in patients with diabetes, a phenomenon described as "aspirin resistance" [1,9]. It has been postulated that acetylation affects the fibrin network structure, making the network more porous [10–12]. A more porous fibrin network has been associated with facilitated fibrinolysis, which may explain part of aspirin's antithrombotic effects [13]. Aspirin seems to dose-dependently increase fibrin network porosity and clot lysis *in vitro* [9,5]. *In vivo* acetylation by high doses of aspirin (650 mg twice daily) shortens the clot lysis time in healthy individuals [5], and increased permeability of fibrin clots has been shown during daily treatment with 37.5–320 mg aspirin in non-diabetic subjects [10–12].

Nonenzymatic glycosylation (glycation) occurs at lysine and valine amino residues in several plasma proteins. In human hemoglobin N-terminal valines and certain lysine amino groups are glycated [14]. Glycation of fibrinogen has been reported to impair fibrinolysis [15,16], and an ameliorated glucose homeostasis in diabetes type I patients results in increased fibrin gel permeability, suggesting formation of a less thrombogenic fibrin network [17] and a detrimental effect of high glucose levels [18]. Interestingly, increased glycation of platelet membrane proteins in diabetes mellitus decreases aspirin-mediated protein acetylation [19]. A similar competition between acetylation and glycation of amino acid residues in albumin has also been reported [20]. Though many of the reports are from *in vitro* experiments using high concentrations of aspirin or glucose, it has repeatedly been shown that oral low-dose

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aspirin treatment leads to an increased fibrin network permeability [10–12,21]. A recently performed cross-over study by our group [22] showed significantly increased fibrin gel permeability during daily treatment with 320 mg in patients with type 1 diabetes, while treatment with 75 mg ASA daily had no effect on fibrin network. This finding could be interpreted as reflecting "aspirin resistance", and that this can be overcome by increasing the aspirin dose.

Clear-cut effects of aspirin have also been reported in a cell- and plasma-free system using recombinant human fibrinogen [23] which is another evidence that that the function of fibrin(ogen) is altered by aspirin. However, it has not been shown at a molecular level how fibrinogen is affected by aspirin, and if acetylation and glycation are interfering processes. Thus, by using mass spectrometry and labeled glucose and aspirin we investigated whether acetylation and glycation occurred at specific amino acid groups in fibrinogen. Our hypothesis was that a) aspirin acetylates fibrinogen at sites critical for fibrin network formation and cross-linking, thus making the network less resistant to fibrinolysis, and b) that glycation also occurs at specific sites in fibrinogen leading to a competition between glycation and acetylation of the protein partly explaining the "aspirin resistance" observed in diabetes.

2. Materials and methods

2.1. Materials

All solvents were of high pressure liquid chromatography (HPLC) grade from Merck, as were trichloroacetic and trifluoroacetic acid; aprotinin (Trasylol®) was from Bayer; aspirin, D-glucose, urea, dithiotreitol, iodoacetamide, ammonium sulphate, ammonium bicarbonate and Spectra/Por disposable dialysis membranes (MW cut-off 8000 Da, 1 mL capacity) were purchased from Sigma–Aldrich. Slide-A-Lyzer dialysis cassettes, 1.0–3.0 mL, were from Pierce Biotechnology, Rockford, USA (10 kDa MW cut-off). Trypsin (TPCK-treated from bovine pancreas) and endoproteinase Glu-C were from Promega Corp. Microcon centrifugal filters (YM-50, MW cut-off 50 kDa) were from Millipore. [^{14}C-acetyl]-ASA (55 mCi/mmol) in toluene and [^{14}C(U)]D-Glucose (300 mCi/mmol) in ethanol:water (10:90, v/v) were purchased from American Radiolabeled Chemicals, Inc. (ARC). Highly purified human fibrinogen was from Sigma (No. 46313-F, 95% of protein clottable, plasminogen and plasmin, each $\leq$0.03 U/mg, as stated by the manufacturer). About 50% by weight was found to be clottable fibrinogen, as determined with our routine Clauss method in a Sysmex CS-2100i instrument; the remaining amount being sodium citrate and sodium chloride. Amounts or concentrations of fibrinogen in text are given as clottable fibrinogen.

2.2. Methods

Fibrinogen incubations and mass spectrometry.

2.2.1. Method 1

Fibrinogen, 2.5 mg/mL (7.35 μM) in 50 mM Tris/100 mM NaCl, pH 7.40, was exposed to ASA (0.8–1.6 mM) or glucose (20–100 mM) in 1.5 mL Eppendorf polyethylene snap vials at 37 °C for 24 h and 5–7 days, respectively, in an Eppendorf Thermomixer (shaking frequency, 450/min). After dialysis over-night, trypsin was added at an enzyme:substrate ratio of 1:50–1:20 and incubation proceeded for 4–8 h at 37°. Peptides were then separated by ultrafiltration at 8000 g for 10 min at +4 °C and mass spectrometry (MS) was performed directly on the ultrafiltrate. Analyses were performed on a MALDI-MS Voyager DE-Pro (Applied Biosystems) with -cyano-4-hydroxycinnamic acid as matrix.

2.2.2. Method 2

Incubates with fibrinogen in buffer, as described in Method 1, were freeze-dried (SpeedVac) and then dissolved in 8 M urea with 0.1 M Tris–HCl, pH 8.0, and reduced with 15 mM dithiotreitol (DTT) at 37° for 45 min. 20–160 μL of clear filtrate were injected in a Kovalent® high-pressure liquid chromatography (HPLC) equipment, using a Phenomenex Jupiter LC-4, 10 μm, 250 × 4.6 mm, reversed phase column. Effluent peaks were monitored at 215 nm, collected and frozen at −70 °C. Phase A was 2.5% acetonitrile (ACN) with 0.05% trifluoroacetic acid (TFA), and phase B was 60% ACN with 0.05% TFA in water. The gradient with phase B started at 5 min before injection, B being 50% at injection (time = 0), and increasing to 80% at 28 min, holding 80% for 10 min. Flow rate was 1 mL/min. For details of the MS analyses, please see Fig. 4.

2.3. Labeling studies

[^{14}C(U)]D-Glucose or [^{14}C-acetyl]ASA were added to fibrinogen dissolved in tris-saline buffer (2.5 mg/mL) and incubated (see Method 1 above). The organic solvents were evaporated just before addition of buffer solutions containing fibrinogen, aspirin, glucose and sodium azide (0.02%), giving the calculated final concentrations. Incubations proceeded for 24 h with aspirin and for 5 days with glucose at 37 °C, if not otherwise stated. After dialysis the total radioactivity remaining in the dialysate was determined in a Packard 1500 Tri-Carb Liquid Scintillation Analyzer using Ultima Gold (Packard Instrument Company, USA) as scintillation medium. The incorporation of labeled groups into fibrinogen were calculated from the total radioactivity, the specific radioactivity, and the measured fibrinogen amount in the dialysate.

To calculate the relative labeling of the α-, β- and γ-chains fibrinogen was incubated with [^{14}C-acetyl]ASA for 24 h at 37°, reduced with 15 mM DTT, freezedried (SpeedVac), dissolved in 8 M urea in 0.1 M Tris–HCl pH 8.0, and separated by HPLC into three peaks which were collected and their contents of radioactivity counted. The activity was corrected for background by subtracting mean baseline background activity counted in ten consecutive fractions (0.5 mL each) collected before the first radioactive peak appeared.

2.4. Glycation, acetylation and competitive binding experiments

Because the peak plasma levels after orally given aspirin in clinical praxis have been reported to be about 10–100 μM, incubations with fibrinogen were carried out with undiluted highly active [^{14}C]ASA (i.e. aspirin), corresponding to about 13–25 μM, as well as with various additions of unlabeled aspirin, covering the presumed plasma range.

(A) Fibrinogen 2.5 mg/mL and 8 mM glucose was mixed with 1.0×10^6 cpm [^{14}C]glucose/mL and incubated at 37° for 10 days with and without 0.8 mM aspirin in 50 mM Tris/100 mM NaCl (pH 7.40, 0.02% sodium azide) in a shaking device protected from light. Two different methods for precipitating the fibrinogen were used, according to Qiu et al. [24], and Day et al. [20]. Aliquots of the incubates were drawn after 0, 1, 2, 4, 6, 8 and 10 days and checked for incorporation of radioactivity into fibrinogen. The aliquots were precipitated with ice-cold trichloroacetic acid 8% and ethanol 10%, final concentrations, respectively.

(B) In another set of experiments 2.5 mg fibrinogen in 1 mL buffer (50 mM Tris/100 mM NaCl, pH 7.40) was incubated with 2×10^6 cpm [^{14}C] glucose in the presence of 0 and 2 mM glucose, with or without 0.8 mM aspirin. Similarly, fibrinogen was incubated with 2×10^6 cpm [^{14}C]ASA with and without 100 mM glucose and with glucose preincubated 48 h before addition of [^{14}C]ASA.

Table 1
Fibrinogen peptide ions with acetyl (+42 Da) or glucose (as ketoamine, +162 Da) adducts at indicated lysines (**K**), found with mass spectrometry after digestion of the fibrinogen chains with trypsin and endoproteinase Glu-C (See under Methods).

	Peptide ion $[M+H]^+$	Start	End	Peptide sequence	Modification (α, β, γ chain)
1	1651.9377	184	197	(K)QLEQVIA**K**DLLPSR(D)	Acetyl K-191 (α)
2	1483.7977	207	219	(K)M**K**PVPDLVPGNFK(S)	Acetyl K-208 (α)
3	1381.75	220	230	(K)SQLQ**K**VPPEWK(D)	Acetyl K-224 (α)
4	1309.66	428	439	(K)E**K**VTSGSTTTTR(R)	Acetyl K-429 (α)
5	1422.7588	449	461	(K)TVIGPDGH**K**EVTK(E)	Acetyl K-457 (α)
6	2800.3630	529	554	(R)GSESGIFTNT**K**ESSSHHPGIAEFPSR(G)	Acetyl K-539 (α)
7	1871.85	557	572	(K)SSSYS**K**QFTSSTSYNR(G)	Acetyl K-562 (α)
8	2299.1042	217	236	(K)GGETSEMYLIQPDSSV**K**PYR(V)	Acetyl K-233 (β)
9	2286.044	131	147	(K)QV**K**DNENVVNEYSSELEK(H)	GlcK-133 (β)
10	1335.94	163	173	(K)QSGLYFI**K**PLK(A)	Acetyl K-170 (γ)
11	1076.7	267	275	(K)VGPEAD**K**YR(L)	Acetyl K-273 (γ)
12	2682.30	63	85	(K)AIQLTYNPDESS**K**PNMIDAATL**K**(S)	GlcK-85 (alt.−75) (γ)

(C) In ten experiments [^{14}C]ASA and [^{14}C]glucose with different specific activities (final concentrations 20, 30, 50, 100 and 200 μM for aspirin and 0.00321, 2, 8, 20 and 100 mM for glucose) was incubated with fibrinogen for 24 h and 5 d, respectively. The molar ratios of incorporated acetyl groups and glucose in fibrinogen were calculated.

(D) In order to study the possible interaction between aspirin and glucose in binding to fibrinogen, twelve experiments were performed to study the effect of simultaneous addition to fibrinogen of [^{14}C]glucose and aspirin; molar ratios were calculated.

(E) To study if preincubation of fibrinogen with glucose would influence the acetylation, in twelve experiments fibrinogen was exposed for glucose (0, 8, 20 and 100 mM) for 5 d at 37 °C, after which [^{14}C]ASA was added (0, 0.05 and 0.8 mM) to give three different specific activities. Incubation proceeded for another 24 h, after which molar ratios of bound acetyl groups to fibrinogen were calculated.

2.5. Dialysis

Dialysis (sample volumes 0.5–1 mL, 24 h, 20 °C) of incubates of fibrinogen with aspirin or glucose were performed with changes of dialysis medium (5 mM Tris/150 mM NaCl, pH 7.40, 600–800 mL) after 2, 4 and 24 h. Dialysis was performed at room temperature with or without proteolysis inhibitor (aprotinin) and 1 mM EDTA in the dialysis medium. These conditions did not influence the HPLC mass peaks or radioactivity recovered in the samples or in the dialysis medium after dialysis. When [^{14}C]ASA was incubated with fibrinogen 10 mM salicylate was added to the dialysing medium in order to "displace" any non-covalently bound [^{14}C-acetyl]ASA.

3. Results

3.1. Acetylation and glycation as assessed by mass spectrometry

3.1.1. Method 1

Using 0.8–1.6 mM aspirin (24 h) and 20–40 mM glucose (24–48 h at 37°) acetylations were found in lysine residues in two peptides from the -chain at positions 208 and 539 (see Table 1). These results were confirmed twice. Fig. 1 shows the LC–MS/MS sequence spectrum of the modified peptide containing AcK (acetylated lysine) 539. No glycations were found at this position. Because of low sequence coverage (25–30%) this method was later modified by sequentially incubating fibrinogen with trypsin (higher ratio 1:20) and endoproteinase Glu-C, using HPLC separated fibrinogen chains instead of intact fibrinogen, and using a high-sensitive LC–MS/MS (Q-TOF Premier API Instrument, Waters Inc.) for sequence analysis (Method 2).

3.1.2. Method 2

Using 0.8 mM aspirin (24 h) and 100 mM glucose (5 d) incubations with fibrinogen, five additional acetylations were found in the α-chain: lysines 191, 224, 429, 457, and 562; in the β-chain lysine 233, and in the γ-chain lysines 170 and 273. Glycations were found at lysine 133 in the β-chain and at lysine 85 (alternatively 75) in the γ-chain. After reduction of fibrinogen the α-, β- and γ-chains were completely separated by HPLC as seen in Fig. 2. A schematic drawing of the fibrinogen molecule showing the found acetylated and glycated sites is depicted in Fig. 3. Data on LC–MS/MS analyses of the peptide fragments after enzymatic degradation of the α-chain by trypsin and Glu-C are shown in Fig. 4. The sequence coverage was 95% for the α-chain, and 90 and 93% for the β- and γ-chain, respectively. The peptides with acetyl and glucose adducts found after trypsin and Glu-C incubations were identified as uncleaved dipeptides and are shown in Table 1.

3.2. Labeling studies

3.2.1. Acetylation of fibrinogen

In five different experiments the molar ratio aspirin/fibrinogen was found to be 0.023–0.041 (mean 0.031) using undiluted labeled aspirin (mass concentration is 22.9–24.7 μM; Table 2a). When 10 μM unlabeled aspirin was added to the labeled one, the resulting ratio was little higher, 0.049, indicating increased acetylation. The relative distribution of radioactivity between the separated fibrinogen chains in three different experiments shows a preponderance for the α-chain (41–51%), and about half of that was observed in the γ-chain (21–24%) (Table 2b).

3.3. Possible competition between glycation and acetylation of fibrinogen

A. A.When using 8 mM glucose the incorporated radioactivity into fibrinogen increased to about 0.08–0.1% of the total amount of radioactivity incubated, irrespective of the presence of 0.8 mM aspirin or not (data not shown).

B. Incubation experiments with fibrinogen performed under the same conditions but with 2 mM glucose resulted in 0.21–0.24 mol glycated lysine residues/mol fibrinogen. Incubations with only the highly labeled (undiluted) aspirin (12.9 μM) or glucose (3.21 μM) resulted in very low molar ratios, i.e. 1.4×10^{-4} and 2.6×10^{-4} respectively (Table 3a).

C. The above mentioned results were confirmed in a series of experiments with varying concentrations of aspirin and glucose (Table 3b–d). We found, with increasing

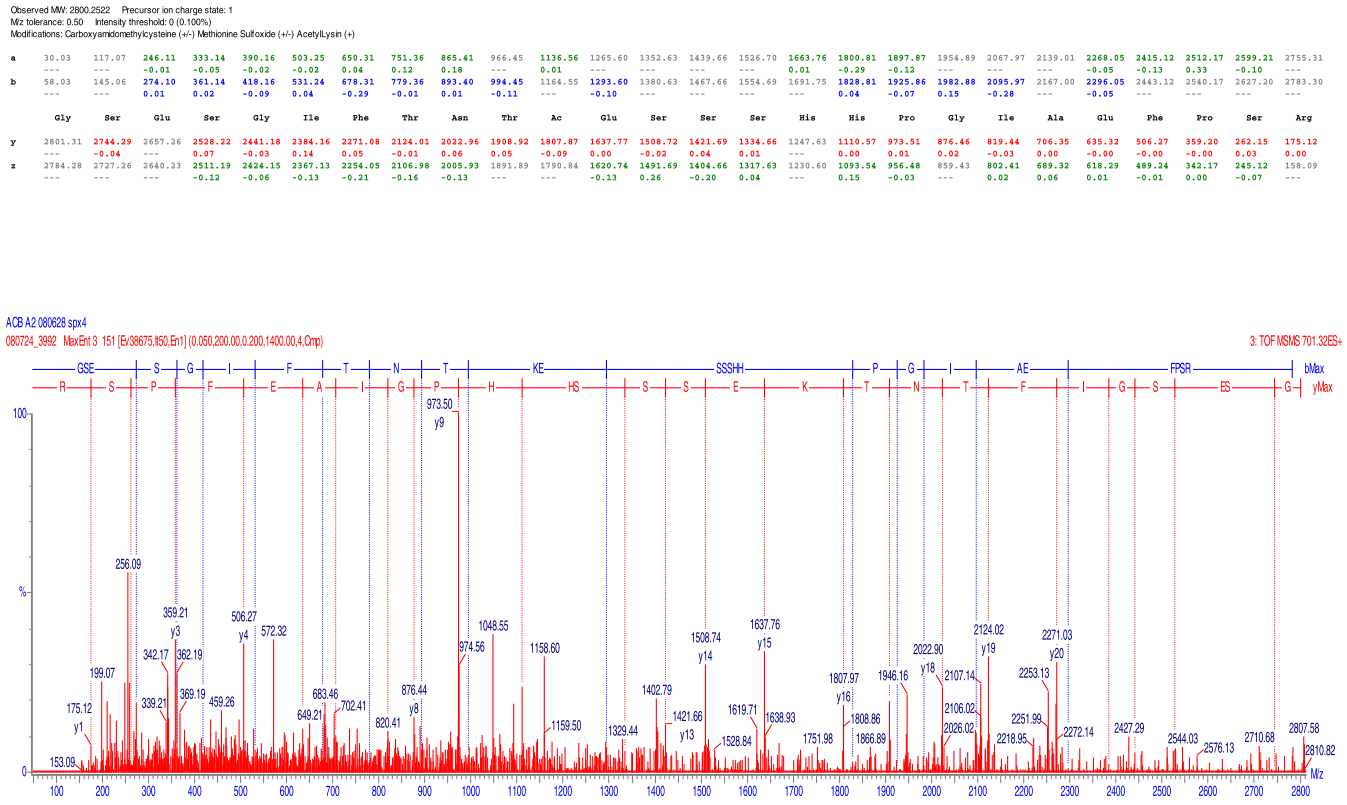

Fig. 1. LC–MS/MS spectrum of the dipeptide T63–64, 26 amino acids, MW 2800.2522 containing the acetylated αK539. Data were acquired with a Q-TOF Ultima or a Waters Q-TOF Premier mass spectrometer using ExPASy data base UniProtKB/Swiss-Prot entries, ProteinLynx and Phenyx-on-line data bases, and Mascot soft ware. The data bases were used for search of generated peptides with mass differences of +162 Da (glucose in ketoamine form) and +42 Da (acetyl).

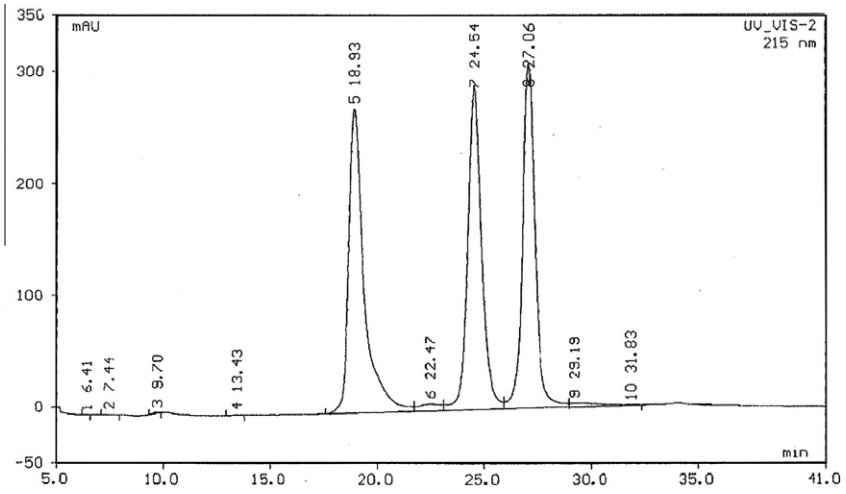

Fig. 2. HPLC separation of the reduced fibrinogen α-,β-, and γ chains (appearing in that order). For details, see under Method 2.

concentrations, a linear increase for molar ratios of acetylated/non-acetylated fibrinogen up to about 2 at 0.8 mM aspirin and, for glycated/non-glycated fibrinogen, there was a linear increase up to about 6.0 (see Table 3b) at 100 mM glucose.

D. As shown in Table 3c we found no support for any influence of aspirin, added in three different concentrations (0, 0.05 and 0.8 mM), on the molar ratios obtained for the different levels of glucose incubated with fibrinogen.

E. Preincubation of fibrinogen for 5 days (Table 3d) with increasing addition of glucose (0, 0.05 and 0.8 mM) did not show any influence of aspirin added at day 5 and incubated for another 24 h at 37 ℃. Fibrinogen determined with a different immunological assay (Immage®, Beckman Coulter Inc.) confirmed these results (data not shown).

4. Discussion

We found about 41% of total incorporated radioactivity in the α-chain, 35% in the β-chain and remaining 24% in the γ-chain of the fibrinogen molecule. This roughly corresponds to the acetylations found by MS (Fig. 3). These numbers are similar to those reported by Bjornsson et al. [5]. They found about 1/3 of total acetylation in each fibrinogen chain, based on counting radioactivity in gel slices

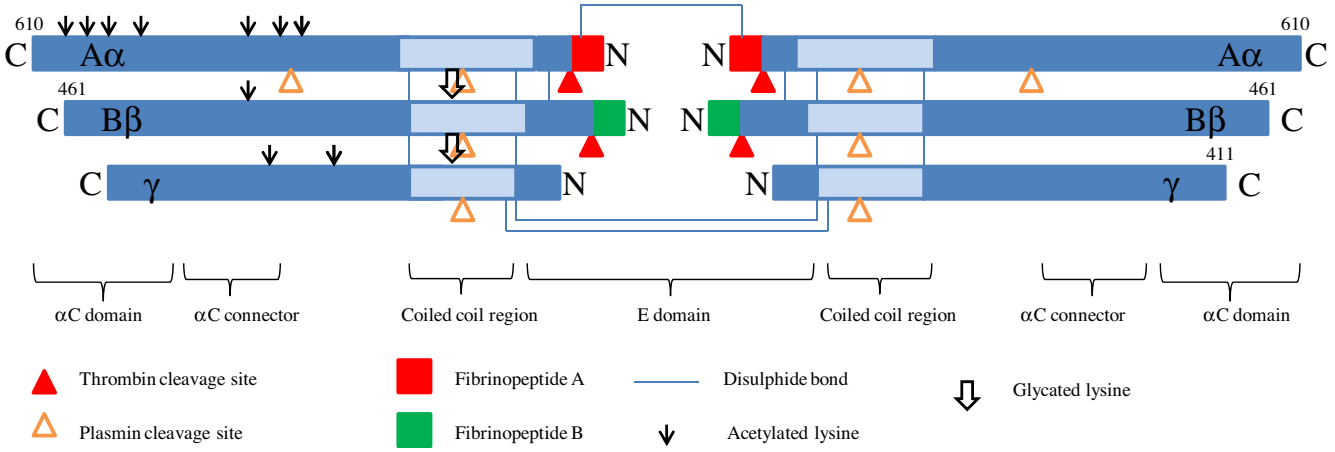

Fig. 3. Schematic diagram of the six polypeptide chains of fibrinogen and found acetylated (black arrows) and glycated (open arrows) lysine amino acid residues (shown only in the left part of the figure). With the exceptions of αK191 and αK208 all the acetylations in the α-chain are within the αC connector (221–391) and αC domain (392–610), which together form the αC region, a mobile part of the α-chain.

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  1 MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS
 51 DWPFCSDEDW NYKCPSGCRM KGLIDEVNQD FTNRINKLKN SLFEYQKNNK
101 DSHSLTTNIM EILRGDFSSA NNRDNTYNRV SEDLRSRIEV LKRKVIEKVQ
151 HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC RGSCSRALAR EVDLKDYEDQ
201 QKQLEQVIAK DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ LQKVPPEWKA
251 LTDMPQMRME LERPGGNEIT RGGSTSYGTG SETESPRNPS SAGSWNSGSS
301 GPGSTGNRNP GSSGTGGTAT WKPGSSGPGS TGSWNSGSSG TGSTGNQNPG
351 SPRPGSTGTW NPGSSERGSA GHWTSESSVS GSTGQWHSES GSFRPDSPGS
401 GNARPNNPDW GTFEEVSGNV SPGTRREYHT EKLVTSKGDK ELRTGKEKVT
451 SGSTTTTRRS CSKTVTKTVI GPDGHKEVTK EVVTSEDGSD CPEAMDLGTL
501 SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF VSETESRGSE
551 SGIFTNTKES SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS
601 YKMADEAGSE ADHEGTHSTK RGHAKSRPVR DCDDVLQTHP SGTQSGIFNI
651 KLPGSSKIFS VYCDQETSLG GWLLIQQRMD GSLNFNRTWQ DYKRGFGSLN
701 DEGEGEFWLG NDYLHLLTQR GSVLRVELED WAGNEAYAEY HFRVGSEAEG
751 YALQVSSYEG TAGDALIEGS VEEGAEYTSH NNMQFSTFDR DADQWEENCA
801 EVYGGGWWYN NCQAANLNGI YYPGGSYDPR NNSPYEIENG VVWVSFRGAD
851 YSLRAVRMKI RPLVTQ
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Fig. 4. Fibrinogen α-chain amino acid sequence showing the combined sequence coverage (95%) obtained from trypsin and endoproteinase Glu-C digestion of fibrinogen and peptide analysis with LC/MS/MS. Matched peptides are shown in bold red. Unidentified sequences are in black. Signal peptide sequence is 1–19 and propeptide is 632–866. Nanospray-ESI MS (electro-spray ionization mass spectrometry) of samples were introduced by needle (Proxeon) in a Q-TOF Ultima mass spectrometer (Waters Inc.). LC–MS/MS (liquid chromatography–mass spectrometry) samples were introduced using nanoAcquity-cLC combined with Q-TOF Ultima or a Q-TOF Premier API Instrument (Waters Inc.). The mass accuracy was 0.005. After drying in vacuum of the samples (40 μL, about 105 μg protein) from HPLC, they were dissolved in 0.2 M ammonium bicarbonate/20% acetonitrile (pH 8) and sonicated for 10 min. After treatment with 5 mM DTT for 30 min at 56 °C and iodoacetamide, 10 mM for 30 min at room temperature, half the sample volume (80 μL) were incubated with trypsin 0.25 μg/μL at an enzyme:substrate ratio of 1:20 (w/w), over night at 37 °C in a shaking mixer. Incubations were stopped by addition of 4 μL formic acid, and 2 μL sample was used for MALDI-MS to check that cleavage had occurred. The residual sample was used for cleavage with endoproteinase Glu-C, 1 μg/μL, over night at pH 8. Reaction was stopped with 5 μL formic acid, purified through StageTip, solved in 150 μL A buffer Orbi (about 0.3 μg/μL), and 5 μL injected into the mass spectrometer. The HPLC columns were Waters dC18 5 μm nanoease Trap column and Waters dC18 column 3 μm, 100 Å; solvent A was 0.1% formic acid/H_2O and solvent B was 0.1% formic acid/acetonitrile, increasing B from 3% to 30% in 30 min.

after reduction and electrophoretic separation. Furthermore, our finding of about 0.02–0.17 mol labeling of fibrinogen by aspirin (at 20–200 μM), are lower numbers than those reported *in vitro* by Bjornsson et al. [5] who found about 3 mol of acetylated lysine residues/mole of fibrinogen. It should be noted that the concentrations of aspirin used in our study (i.e. 12.9–200 μM) cover the therapeutic plasma concentration range. Earlier work report peak plasma levels of about only 5.6–17 μM aspirin 30 min after single oral doses of 80–320 mg [26,27]. Two hours after the last dose of aspirin at 650 mg twice daily a plasma concentration of about 9 μM of aspirin was reported by Bjornsson et al., whereas peak values were around 50–60 μM (10 μg/mL) [5]. Low-dose aspirin treatment would thus probably result in only a few percent of acetylation of fibrinogen, on a molar basis, to judge from results obtained in our *in vitro* labeling studies (.e.g. 4.3% labeling at 50 μM aspirin, Table 3b).

Using a peptide probing technique Sobel et al. [28] found the following twelve α-chain lysines having cross-linking activity: lysines 208, 224 and/or 219, 418, 427, 429, 448, 508, 539, 556, 580, 601 and 606. Interestingly, Lys-539 is one of several recognized lysines in the α-chain that function as donor lysines in FXIII cross-linking (Fig. 3). Moreover, the lysines 418, 508, 539, 556 and 601 were recently verified as cross-linking sites by mass spectrometry, with a method also identifying the corresponding

Table 2
(A) Binding (molar ratios) of highly active [^{14}C-acetyl]ASA to fibrinogen incubated *in vitro* (24 h, 37 °C), calculated from radioactivity remaining after dialysis. (B) Relative (%) distribution of ASA radioactivity of the fibrinogen α-, β- and γ-chains, isolated as HPLC peaks (mean of $N = 3$).

	Molar concentration (μM) of ASA incubated		
A. Molar ratio of acetylated fibrinogen/total fibrinogen			
0.029	22.9		
0.029	22.9		
0.041	24.7		
0.024	24.1		
0.032[a]	23.7		
0.030[b]	24.7		
0.049[c]	32.7		
	Mean ($N = 3$)	Sodium salicylate, 10 mM	ASA, 10 μM
B. Percent radioactivity in HPLC peaks			
Peak (α)	41	45	51
Peak (β)	35	32	28
Peak (γ)	24	22	21

[a] Preincubation for 48 h, 37 °C.
[b] Sodium salicylate (10 mM) was added to dialysis medium in one experiment.
[c] 10 μM ASA was added to the fibrinogen 24 h incubation mixture.

cross-linked glutamine residues in native fibrin [29]. Lys-539 and Gln-237 were found to be the two amino acid residues that were the most important ones involved in cross-linking, with lys-539 binding to four different α-chain glutamines. Our finding of acetylation of Lys-539, Lys-429, Lys-224 and Lys-208 may therefore have potential implications for FXIII cross-linking and the final fibrin structure that is formed in the presence of acetylated fibrin(ogen). The functional significance of acetylation of the other amino acid residues found in our study is, as far as we know, unknown.

Glycation of the fibrinogen molecule was also investigated by MS. We found two glycated lysines (GlcK-133 in the β-chain and GlcK-75 (alt. 85) in the γ-chain), and these are within the "plasmin-sensitive" coiled–coil regions, GlcK-133 being only two amino acids separated from one of the two known sites of plasmin proteolysis (K-130). If these glycations will interfere with fibrinolysis is presently not known. Regarding the labeling experiments, we found 0.12–0.5 mol glycated residues bound/mole fibrinogen when incubation was performed with concentrations in the physiological range (i.e. 2–8 mM) (Tables 3a and 3b). With 20 and 100 mM glucose molar ratios of about 2 and 10, respectively, were found (Tables 3b and 3c). These data are roughly in agreement with those reported by Mirshahi et al. [30], Austin et al. [31], Lütjens et al. [32] and by Pieters et al. [33]. Thus, these authors found glycation *in vitro* and *in vivo* of 0.95–8 mol glucose/mole fibrinogen. Glucose adducts at the two or possible three positions found for glucose in this study would theoretically allow for binding of maximally 4–6 mol of glucose/mole fibrinogen.

Ajjan et al. [23] have shown that aspirin (at 0.55 mM) exerts the same effects on purified recombinant human fibrinogen as on ex vivo purified fibrinogen from aspirin-treated volunteers (i.e. it causes acetylation, increased clot permeability, looser clot structure, increased fibrinolysis and dose-dependent increase in turbidity/fibrin polymerization, indicating thicker fibrin fibers). This implies that fibrin(ogen) is a specific effector molecule for all these aspirin influences. However, additional effects may add *in vivo* as other coagulation proteins in plasma may be acetylated as well [6,7,25]. Of note, aspirin is much more reactive than glucose in forming adducts on free amino groups, as can be seen from Table 3d, where 0.8 mM aspirin generates 2.0–2.5 mol bound, sim-

Table 3a
Incubations (1–4) of fibrinogen (2.5 mg/mL) with [^{14}C]glucose (0 and 2 mM) in Tris-saline buffer pH 7.40 at 37 °C for 5 days with and without 0.8 mM ASA and incubations (5–7) of [^{14}C]ASA with and without 100 mM glucose. In sample No. 7 glucose was added 48 h before the addition of [^{14}C]ASA in order to investigate if preincubation of glucose would influence the binding of [^{14}C]ASA. The mass concentration of glucose in experiments 1–2 is 3.21 μM and of ASA in experiments 5–7 is 12.9 μM.

No.	Incubations of fibrinogen in experiments 1–7	Cpm remaining in dialysate	mg Fibrinogen in dialysate	Moles of glycated or acetylated lysine residues/mole fibrinogen
1	[^{14}C]glucose + 0 mM ASA	240	0.93	1.4×10^{-4}
2	[^{14}C]glucose + 0.8 mM ASA	319	0.66	2.5×10^{-4}
3	[^{14}C]glucose + 2 mM glucose + 0 mM ASA	294	0.48	0.21
4	[^{14}C]glucose + 2 mM glucose + 0.8 mM ASA	416	0.66	0.24
5	[^{14}C]ASA + 0 mM glucose	9392	0.82	0.0251
6	[^{14}C]ASA + 100 mM glucose	12956	0.78	0.0365
7	[^{14}C]ASA + 100 mM glucose (preincub. 48 h)	12417	0.86	0.0317

Table 3b
Incubations of fibrinogen (2.5 mg/mL) with [^{14}C-acetyl]ASA, 2×10^6 cpm (20 μM ASA), and [^{14}C]glucose, 2×10^6 cpm (3.21 μM glucose), with increasing concentrations of unlabeled ASA or glucose, and resulting molar ratios calculated after dialysis.

Sample No.	Addition of unlabeled ASA (μM)	Final concentration of ASA (μM)	Cpm in dialysate	Total fibrinogen (mg in dialysate)	Molar ratio of acetylated fibrinogen/total fibrinogen
ASA					
1	0	20	10890	1.44	0.017
2	10	30	9507	1.24	0.025
3	30	50	10319	1.31	0.043
4	80	100	10925	1.42	0.084
5	180	200	10426	1.36	0.17
Sample No.	Addition of inactive glucose (mM)	Final concentration of glucose (mM)	Cpm in dialysate	Total fibrinogen (mg in dialysate)	Molar ratio glycated fibrinogen/total fibrinogen
Glucose					
1	0	0	306	0.817	0.000204
2	2	2	333	0.940	0.12
3	8	8	350	0.952	0.50
4	20	20	439	0.869	2.03
5	100	100	382	1.070	6.07

Table 3c
Competition experiments: Simultaneous incubation of [^{14}C]glucose and ASA with fibrinogen to study the effect of ASA on the incorporation of glucose at different glucose levels. Three different levels of ASA were used for each level of glucose.

Sample No.	Incubation of fibrinogen 5 mg/mL in Tris–NaCl (50 + 100 mM), pH 7.40 + 2 × 10^6 cpm[a]	Addition of glucose (mM)	ASA (mM)	Total cpm in dialysate	Total fibrinogen mg in dialysate	Molar ratio glycated fibrinogen/total fibrinogen
1	Fib. +[^{14}C]glucose	0	ASA 0	276	0.43	4.4×10^{-4}
2	D:o	0	ASA 0.05	295	0.39	4.2×10^{-4}
3	D:o	0	ASA 0.8	344	0.33	5.6×10^{-4}
4	Fib. +[^{14}C]glucose	8	ASA 0	293	0.61	0.65
5	D:o	8	ASA 0.05	297	0.49	0.82
6	D:o	8	ASA 0.8	366	0.48	1.1
7	Fib. +[^{14}C]glucose	20	ASA 0	386	0.52	2.5
8	D:o	20	ASA 0.05	401	0.43	3.2
9	D:o	20	ASA 0.8	336	0.62	2.2
10	Fib. +[^{14}C]glucose	100	ASA 0	448	0.49	15.4
11	D:o	100	ASA 0.05	386	0.68	9.7
12	D:o	100	ASA 0.8	279	0.48	9.9

[a] Addition of [^{14}C]glucose, 2 × 10^6 cpm, implies the addition of 3.21 μM ASA (calculated from the specific activity of 300 mCi/mmol).

Table 3d
Competition experiments: Preincubation of fibrinogen with glucose 0–100 mM during 5 days to first generate glycated fibrinogen. Thereafter [^{14}C]ASA was added and incubation proceeded for 24 h, to investigate if acetylation was inhibited. Three different ASA levels (i.e. specific activities) were chosen. As shown, the addition of glucose did not influence the acetylation caused by ASA.

Sample No.	Preincubation of fibrinogen 5 mg/mL Tris–NaCl, pH 7.40	[^{14}C]ASA added after 5 d of incubation[a]	Addition of unlabeled ASA (mM)	Total cpm in dialysate	Total fibrinogen (mg in dialysate)	Molar ratio acetylated fibrinogen/fibrinogen
13	Fib + Glucose 0 mM	2 × 10^6 cpm	0	5599	0.98	0.013
14	Fib + Glucose 8 mM	2 × 10^6 cpm	0	4579	0.78	0.013
15	Fib + Glucose 20 mM	2 × 10^6 cpm	0	7186	1.12	0.014
16	Fib + Glucose 100 mM	2 × 10^6 cpm	0	6891	0.92	0.016
17	Fib + Glucose 0 mM	2 × 10^6 cpm	0.05	6227	1.15	0.058
18	Fib + Glucose 8 mM	2 × 10^6 cpm	0.05	6782	1.04	0.070
19	Fib + Glucose 20 mM	2 × 10^6 cpm	0.05	9789	0.53	0.20
20	Fib + Glucose 100 mM	2x10^6 cpm	0.05	11,597	0.55	0.23
21	Fib + Glucose 0 mM	2 × 10^6 cpm	0.8	10,425	0.58	2.47
22	Fib + Glucose 8 mM	2 × 10^6 cpm	0.8	4898	0.33	2.0
23	Fib + Glucose 20 mM	2 × 10^6 cpm	0.8	10,895	0.71	2.1
24	Fib + Glucose 100 mM	2 × 10^6 cpm	0.8	10,323	0.58	2.5

[a] Addition of [^{14}C]ASA, 2 × 10^6 cpm, implies the addition of 0.0129 mM ASA (calculated from the specific activity of 50 mCi/mmol).

ilar to what is bound in the presence of 20 mM glucose during the same incubation period. A similar finding has been reported for platelet membrane proteins [19].

We cannot find any interaction between aspirin and glucose in binding to fibrinogen, nor would it be expected, because aspirin and glucose bind to different lysine sites, as shown by our MS data. Our study is, as far as we know, the first to show which specific lysine residues in fibrinogen that are acetylated and glycated by aspirin and glucose, respectively. Furthermore, we have also determined the amounts of the adducts bound to fibrinogen at plasma concentrations of glucose and aspirin considered to be physiologically and clinically relevant. Notably, several of these sites are of potential importance for cross-linking by FXIIIa [28,29], and may thus also influence the fibrin network. Further studies are needed to fully understand the relevancy of the present findings to explain the *in vivo* effects of aspirin and glucose on the fibrin network.

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