Effect of Aspirin on Determinations of Glycosylated Hemoglobin

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We investigated the in vivo and in vitro effects of aspirin on several clinical assays of glycosylated hemoglobin. Acetylation of hemoglobin falsely increased the glycosylated hemoglobin fraction measured by "high-performance" liquid chromatography and electrophoresis, but isoelectric focusing and colorimetric techniques differentiated between acetylated and glycosylated fractions. Aspirin ingestion may result in an apparent increase in glycosylated hemoglobin measured with common clinical assays.

Additional Keyphrases: variation, source of · diabetes · chromatography, liquid · electrophoresis · isoelectric focusing · colorimetry · differentiation of glycosylated and acetylated hemoglobin · acetylsalicylic acid

The measurement of glycosylated hemoglobin concentrations by chromatographic methods is adversely affected by uremia (1), lipemia (2), and hemoglobinopathies (3). Aspirin (acetylsalicylic acid) is cited as a drug that interferes with the determination of glycosylated hemoglobin (4), although no published studies have evaluated its effect on the commonly used clinical assays. Aspirin has many similarities to glucose; it has the same molecular mass, causes post-translational modification of lysine residues, and confers a negative charge on the modified protein (5). We investigated the acetylation of hemoglobin by aspirin in vitro and in vivo and studied the resulting interference with glycosylated hemoglobin assays.

Materials and Methods

Materials

Acetylsalicylic acid, 2-thiobarbituric acid, and β-D-(+)-fructose were purchased from Sigma Chemical Co., St. Louis, MO 63178; [3H]acetic anhydride from Amersham, Arlington Heights, IL 60005; bisacrylamide from Bio-Rad Laboratories, Richmond, CA 94804; and Ampholine, pH 6–8, from LKB Producter, Bromma, Sweden. Syringe filters (0.45 μm) were from Millipore Corp., Bedford, MA 01730.

Assays

Glycosylated and acetylated hemoglobin fractions were measured by "high-performance" liquid chromatography (HPLC) (6), an electrophoresis method (Corning Medical and Scientific, Palo Alto, CA 94306) (7), isoelectric focusing (8), and a colorimetric assay (9) according to previously published methods with the following modifications. To isolate and purify the hemoglobin A₀ and hemoglobin A₁c fractions that were used in the in vitro incubations, we used HPLC with a 16 cm × 9 mm (i.d.) column. The isolated hemoglobin A₀ and hemoglobin A₁c fractions were at least 99% pure.

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For isoelectric focusing, we used 0.4 × 10 cm rod gels, NaOH (20 mmol/L) catholyte, and phosphoric acid (10 mmol/L) anolyte. We applied approximately 300 μg of protein in a 25-μL volume to the gel. The current was 1 mA per tube and the voltage was maintained at 500 V. The assay was performed at 4 °C.

Unlike the other methods, the colorimetric assay is specific for glycosylated and not acetylated hemoglobin (9). We adjusted the concentration of hemoglobin in the assay to 20 g/L to provide reproducible colorimetric results and included a fructose standard with each run. After incubation of hemolyte with oxalic acid, 0.5 mol/L, in an autoclave (124 °C, 124 kPa) for 1 h, we cooled the solution in ice, precipitated protein with trichloroacetic acid (400 g/L), and filtered the solution through a 0.45-μm (pore size) syringe filter. The interassay coefficient of variation based on eight repeated assays was <5% for samples from non-diabetics and <4% for samples from diabetics.

Hemoglobin Acetylation

Whole blood was collected into EDTA-containing tubes. Erythrocytes were washed twice in isotonic saline and then incubated in 10 volumes of isotonic saline for 14 h at 22 ºC to remove the labile glycosylated fraction before hemolysis (6). Acetylated hemoglobin was generated in vitro by incubating either erythrocytes, hemolyzate, or purified hemoglobin A₀ or A₁c (1 g/L) with acetylsalicylic acid, 0.1 to 14 mmol/L, at 38 ºC for 0.5 to 24 h. Besides the hemoglobin and acetylsalicylic acid, the incubation medium contained phosphate buffer (10 mmol/L, pH 6.9) prepared from NaH₂PO₄ and Na₂HPO₄. A control incubation, without acetylsalicylic acid, was performed under otherwise identical conditions.

After acetylation, the hemoglobin was assayed by HPLC, electrophoresis, isoelectric focusing, or the colorimetric assay.

To determine the site of acetylation on the minor hemoglobin fractions, hemoglobin A₀ was acetylated in vitro with [3H]acetylsalicylic acid, which was prepared by acetylating salicylic acid with [3H]acetic anhydride by the method of Roth and Majerus (5). After incubation of the salicylic acid and [3H]acetic anhydride (2 h, 37 °C), the reaction was stopped by dilution with acetonitrile/acetic acid/water (25/5/70 by vol).

The [3H]acetylsalicylic acid was purified by HPLC chromatography in the above solvent at a flow rate of 1 mL/min on a C₁₈ analytical column (Altex Scientific Inc., Berkeley, CA 94710) (10). The acetylsalicylic peak, monitored at 275 nm with a flow-through spectrophotometer (Model 100-40; Hitachi Instruments, Tokyo, Japan), was collected, evaporated under reduced pressure, and stored at −80 °C. No hydrolysis of this sample was detected by HPLC after storage for two months.

To prepare the [3H]acetyl hemoglobin, we incubated hemoglobin A₀ (0.5 mmol/L) with the [3H]acetylsalicylic acid (4 mmol/L; specific activity 1.44 × 10⁴ cpm/mol) at 38 ºC for 20 h. Aliquots of 250 μL were taken at 2-h intervals and applied to a G-25 Sephadex column to quench the acetylation reaction and separate the unreacted [3H]acetylsalicylic acid and hemoglobin. One-milliliter fractions were collected.

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and the radioactivity of an aliquot of each was counted in 3 mL of scintillation fluid (Aquasol; New England Nuclear, Boston, MA 02118) with a liquid scintillation counter (Hewlett Packard, Downers Grove, IL 60515). The fractions with the highest hemoglobin concentration were pooled and assayed by HPLC; the radioactivity of 400-μL fractions collected from the HPLC column was counted in 3 mL of scintillation fluid.

Studies on Patients

A normal, nondiabetic volunteer ingested 600 mg of aspirin daily for three weeks and 2.4 g daily for four weeks. We assayed hemoglobin A1c by HPLC at two-week intervals during this period. We also studied 23 subjects with rheumatoid arthritis, no history of diabetes, and normal values for plasma glucose during fasting, who had ingested least 4 g of aspirin daily for three months. EDTA-anticoagulated blood samples were obtained and assayed for glycosylated hemoglobin by the HPLC and colorimetric method, and for plasma salicylate concentration (11).

Results

The addition of acetylsalicylic acid (final concentration 4.6 mmol/L) directly to the hemolysate prepared for HPLC or electrophoretic assay did not alter the results (data not shown). Incubation of washed erythrocytes, hemolysate, or hemoglobin A0 with various concentrations of acetylsalicylic acid, however, produced an apparent increase in the glycosylated fraction as measured with HPLC or electrophoresis, an increase that was time- and concentration-dependent (Figure 1). The qualitative profile of the acetylated product was indistinguishable from glycosylated hemoglobin (Figure 2).

Fig. 2. HPLC profiles of purified hemoglobin A0 (0.9 mmol/L) acetylated by incubation with acetylsalicylic acid, 4 mmol/L, 38 °C
Chromatographic recordings of acetylated hemoglobin A0 were identical to that for glycosylated hemoglobin (graphs reproduced from HPLC tracings)

Induction of hemoglobin A0 with [3H]acetylsalicylic acid revealed that incorporation of the [3H]acetyl group was predominantly confined to the fractions represented by the minor peaks. There was 10-fold greater incorporation (moles of acetylsalicylic acid per mole of hemoglobin) into these minor fractions than into the hemoglobin A0 fraction (Figure 3). The calculated incorporation of tritiated acetate into the fast-migrating peaks was approximately 2 mol per mole of quatrimeric hemoglobin.

There was a slight but appreciable increase of glycosylated hemoglobin, from 4.7 to 5.0% and 5.0 to 5.4%, during the period when the normal volunteer ingested 0.6 and 2.4 g of aspirin daily, respectively. Despite the absence of any history of diabetes and the normal glucose concentrations,
Fig. 3. HPLC profile of [3H]acetated hemoglobin generated in vitro and radioactive incorporation of [3H]acetate into hemoglobin A0. HPLC-purified hemoglobin A0 (0.5 mmol/L) was incubated for 6 h with [3H]acetetyl salicylic acid (4 mmol/L) at 38 °C (see text).

13 (57%) of the subjects with rheumatoid arthritis had values for glycosylated hemoglobin in the diabetic range, and 91% had values exceeding 1 SD above the non-diabetic means with the HPLC assay (mean 4.98, SD 0.67). There was no correlation between plasma salicylic acid concentration and the size of the fast peaks. The colorimetric assay, which measures only the glycosylated fraction, showed values for all of the subjects except one that were within the normal range (Figure 4).

Discussion

The measurement of glycosylated hemoglobin has assumed an increasingly important role in the management of diabetes. The vagaries of the clinical assays have been defined and refined. On theoretic grounds, the acetylation of hemoglobin by aspirin would be expected to interfere with clinical assays that separate glycosylated hemoglobin on the basis of charge. Transacetylation of albumin (12), the erythrocyte membrane (13), and hemoglobin (14), all of which are also glycosylated in vivo, has been demonstrated by others. Acetylation of hemoglobin has been investigated as a means of reducing sickling in sickle cell anemia. Although aspirin did not prove to be an effective anti-sickling agent, Shamsuddin et al. showed that lysines on the α and β hemoglobin chain are the major sites of acetylation (15). The N-terminal valine, a major site of glycosylation, does not appear to be a site of acetylation. Earlier work with isoelectric focusing showed that acetylated hemoglobin migrates cathodically from adult hemoglobin, but whether it could be separated from the acidic glycosylated fraction was not noted (14).

In the current work, we have demonstrated that acetylation of hemoglobin by aspirin in vitro produces a minor fraction that is indistinguishable from glycosylated hemoglobin with the HPLC or electrophoresis assays. These assays that rely on the alteration of charge at a fixed pH to separate the modified from native protein make no distinction between an acetylated and glycosylated protein. Isoelectric focusing, on the other hand, separates proteins on the basis of isoelectric point. Since the pKₐ of acetylated lysine differs from that of the glycosylated lysine or valine, acetylated and glycosylated fractions can be differentiated by isoelectric focusing.

We have also demonstrated that ingestion of aspirin in high doses can cause a spurious increase in glycosylated hemoglobin when measured by HPLC or electrophoresis. The lack of correlation between plasma salicylate concentrations and the HPLC measurement probably reflects the inability of a single salicylate measurement to measure long-term patient compliance with aspirin therapy. Measurement of acetylated hemoglobin might prove to be more useful than measurement of plasma salicylate as an index of chronic aspirin ingestion.

Of note, it has been shown that aspirin can decrease glycosylation of serum proteins during in vitro incubation, presumably through site competition (16). Whether acetylation decreases membrane glycosylation in vivo remains to be seen. However, if glycosylation represents a pathogenic mechanism in diabetic vascular disease and if acetylation does decrease glycosylation, this interaction may explain the clinical observation that diabetics on high doses of aspirin have fewer vascular complications (17).

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References


Usefulness of Serial Determinations of Myoglobin and Creatine Kinase in Serum Compared for Assessment of Acute Myocardial Infarction

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Twenty-one patients with their first myocardial infarction underwent serial blood sampling every 2 h for determination of serum creatine kinase (CK) and myoglobin during the first 48–72 h after onset of pain. The first blood sample, obtained at a mean time of 4.4 h after infarct onset, invariably showed increased myoglobin (mean, 8.3-fold normal), whereas CK was often normal (mean, 1.6-fold normal). Peak myoglobin values occurred earlier than peak CK values (9.9 h vs 21.6 h, p < 0.0005), but there was a significant correlation of peak values (myoglobin = 0.384CK – 0.264, r = 0.794, p < 0.0005). The mean exponential disappearance rate (Kd) of CK was 0.00106 min⁻¹ and of myoglobin was 0.00265 min⁻¹ (p < 0.0005). The disappearance of myoglobin was well described by a mono-exponential expression except in two patients. The total duration of the increase in myoglobin was significantly less than that of CK (34.7 h vs 74.4 h, p < 0.0005).

Additional Keyphrases: assessing size of infarct • disappearance rate of infarct markers

Myoglobin is a low-molecular-mass (17,800 Da) heme protein found as a normal constituent of human cardiac and skeletal muscle. It is released by necrosis of these muscles and, when present in large amounts, has been detected in urine by colorimetry (1), and more recently by immunological techniques (2, 3). Quantification of myoglobin in serum after myocardial infarction, first attempted by Kagen et al. (4), is now done with much greater sensitivity by radioimmunoassay (5–8). Myoglobin is present in low concentra-

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