BRIEF COMMUNICATION

ACETYLATION OF ALBUMIN BY LOW DOSES OF ASPIRIN

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Introduction

Aspirin has a variety of pharmacologic actions, which are expressed at different doses of the drug. An effect on platelet function occurs at very low doses of aspirin (1,2). Indeed, a large number of clinical trials have been carried out to assess whether low to moderate doses of aspirin (180 to 1500 mg per day) taken prophylactically will affect the natural history of a variety of diseases in which thrombosis is thought to play a role (3).

High doses of aspirin - similar to those taken to achieve an antiinflammatory effect - have been demonstrated to acetylate plasma albumin, both in vivo (4) and in vitro (5,6). Acetylation by aspirin alters albumin's ability to bind some drugs, such as phenylbutazone and flufenamic acid (7), and may lead to a reduced survival in plasma of prostaglandins (8) and thromboxane A2 (9). Such alterations in the ability of albumin to transport drugs and other substances may have important consequences if a large proportion of the population were taking low or moderate doses of aspirin prophylactically. We, therefore, undertook the following study to evaluate the acetylation of plasma proteins at low doses of aspirin.

Methods

A pool of plasma anticoagulated with 5.8 mM ethylenediaminetetraacetic acid was collected during the isolation of human platelets (10). This pool was frozen in aliquots and used in the following studies. A sample of pooled human serum was obtained from Dr. Peter Nowell. Purified albumin preparations were obtained from Worthington Biochemical and Sigma Chemical. Indomethacin and salicylic acid were obtained from Sigma Chemical. \[^{3}H\text{-acetyl}]\text{aspirin} (specific activity 200 mCi/m mole), was prepared by the method of Roth and Majerus (11). \[^{3}H\text{-Toluene} \text{was purchased from Amersham Radiochemicals.}\[^{14}C\text{-carboxyl}]\text{aspirin} (specific activity 33 mCi/m mole) was purchased from New England Nuclear. Aminohexyl Sepharose 4B was purchased from Pharmacia.

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Ampholines, pH 3-10, were obtained from LKB Broma. Rabbit anti-human albumin was purchased from Calbiochem-Behring. All other chemicals were of reagent grade.

Prior to acetylation plasma was dialyzed against Dulbecco's phosphate buffered saline (PBS, 12) for 24 hours. Purified albumins were acetylated as solutions in PBS. Plasma of albumin was mixed with aspirin at 37° C. At appropriate intervals samples were taken and were made 4% in sodium dodecyl sulfate and 0.05 M in 2-mercaptoethanol. These samples were heated to 100° C for 5 minutes and subjected to polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (13). Tritium incorporation into proteins was quantitated by a previously published method (2). Activities were estimated using 3H-toluene as an internal standard.

Isoelectric focusing was carried out using the method of Bauuann and Chrambach (14). Proteins were acetylated in 0.01 M phosphate, pH 7.0, and were focused on 5% T, 15% C acrylamide gels crosslinked with N,N'diallyltartardiamide in the presence of 2% NP-40. The pH of each gel was measured with a microelectrode, and radioactivity was quantitated as described (2). Affinity columns were prepared by the method of Cambiaso et al (15), using 0.25% glutaraldehyde in 0.1 M sodium carbonate/bicarbonate, pH 8.5. Protein concentrations were assayed by the method of Lowry, et al (16), with bovine serum albumin as standard. Albumin was measured by densitometry following cellulose acetate electrophoresis. Fatty acids bound to purified albumin were assayed by the method of Evenson and Deutsch (17), using stearic acid as standard.

Results

The incubation of serum or plasma with [3H-acetyl]aspirin results in one major peak of radioactivity following SDS-PAGE (Fig. 1). Similar incubations of plasma with [14C-carboxyl]aspirin yield no radiolabeled proteins following SDS-PAGE and indicate that the [3H-acetyl]aspirin is acetylating a protein or proteins.

**FIGURE 1**

Tritium incorporation into plasma protein following incubation with 100 μM aspirin and electrophoresis as described in Methods. Counts per minute (CPM) per 2 mm gel slice are plotted versus gel slice, numbered from top of gel.
in plasma. Solutions of purified albumin acetylated under similar conditions demonstrated an indistinguishable pattern of radioactivity following SDS-PAGE. Furthermore, when samples of serum were acetylated and subjected to isoelectric focusing in the presence and absence of 9 M urea, only a single band of radioactivity was demonstrated with pI identical to that of a similarly acetylated purified albumin control. Occasionally a small band of radioactivity was seen near the top of our SDS-PAGE gels (Fig. 1). This was interpreted as representing albumin dimers, as only one acetylated protein was present on the isoelectric focusing gels. Samples of acetylated plasma were also chromatographed on aminohexyl Sepharose columns coupled with rabbit antihuman albumin or normal rabbit serum. Greater than 90% of the radioactivity in plasma bound to the antihuman albumin column and was subsequently eluted with 3M sodium thiocyanate and 5M guanidine hydrochloride. Only 10% of the radioactivity in plasma was bound by a column prepared with normal rabbit serum. We conclude that albumin is the only plasma protein acetylated in plasma under these conditions.

The rate of acetylation of dialyzed plasma or comparable concentrations of albumin is illustrated in Figure 2. The rate is not linear, but does not conform to that expected of a second order irreversible reaction. This may be

![Diagram](image-url)

**FIGURE 2**

Rate of albumin acetylation by 100 μM aspirin. A: dialyzed plasma; B: purified albumin, 32 mg/ml in PBS.
due to the fact that more than one site is being acetylated. After exposure to 100 μM aspirin for 90 minutes, approximately 2% of the albumin present in plasma is acetylated. We observed only small differences between dialyzed plasma, serum, and purified albumins with respect to the rate or extent of acetylation under these conditions. Specifically, there was no difference in acetylation of fatty acid free albumin versus crystallized albumins containing varying amounts of bound fatty acid.

Acetylation of albumin in plasma is a saturable process. A 0.1% solution of plasma in PBS treated with [3H-acetyllaspirin reached a maximum acetylation of 1.5 moles acetyl residues per mole albumin at 180 minutes, and did not change over the following 24 hours. A solution of purified albumin treated in a similar fashion reached a maximum acetylation of 1.8 moles acetyl residues per mole albumin. Furthermore, a ten-fold excess of unlabeled aspirin inhibits tritium uptake in plasma by 94% (Fig. 3). The concurrent presence of 100 μM indomethacin causes a 96% inhibition of the acetylation of dilute plasma by 100 μM aspirin (Fig. 3).

**FIGURE 3**

Inhibition of tritium incorporation by excess unlabeled aspirin or by indomethacin. Plasma was diluted in PBS to 2% (v:v) and treated with 100 μM radioactive aspirin. Squares: radioactive aspirin alone; circles: radioactive aspirin plus 10 mM unlabeled aspirin; triangles: radioactive aspirin plus 100 μM indomethacin.

**DISCUSSION**

Following ingestion of 650 mg aspirin, plasma aspirin levels peak at approximately 120 μM 20 minutes after ingestion and fall to undetectable levels by 120 minutes (18). We have studied the in vitro acetylation of plasma proteins using 100 μM aspirin over 90 minutes to approximate plasma exposure to aspirin in vivo during prophylactic therapy with small daily doses of aspirin. Plasma was dialyzed against PBS to avoid changes in pH during in vitro acetylation, as changes in pH markedly alter the rate of acetylation of amino groups (19). Although a number of plasma proteins and other macromolecules are acetylated by aspirin under more rigorous conditions (20), only albumin is acetylated under the conditions of our study. It is interesting that the concurrent presence of 100 μM indomethacin inhibits the rate of acetylation of albumin in plasma by 96%. This suggests that these drugs bind
to related sites on albumin. One may also note from the present study that a biological effect shared by aspirin and indomethacin should not be interpreted a priori as an effect on fatty acid cyclooxygenase, as the drugs share other characteristics besides their ability to inhibit this enzyme.

The rate of albumin acetylation we measure would be sufficient to acetylate albumin completely in approximately two months of daily low dose aspirin ingestion, assuming an albumin half life of 17 days and no preferential metabolism of acetylated albumin. Since certain drugs bind differently to acetylated albumin, albumin's function as a transporter of anions may be altered in patients on aspirin prophylaxis. The half life of thromboxane A₂ is much shorter in the presence of acetylated albumin than in the presence of native albumin (9). Furthermore prostaglandins also bind to albumin, and this binding may be reduced following aspirin therapy (8). Therefore, chronic aspirin therapy may exert an antithrombotic effect by altering plasma thromboxane/prostaglandin ratios as well as acting as an antiplatelet agent.

It is doubtful that chronic low-dose aspirin ingestion will result in any serious toxicity. However, there may be detrimental effects of chronic aspirin ingestion that are currently unrecognized (21). The recognition of aspirin's effects, such as its ability to acetylate albumin, may be important in the evaluation of patients using the drug as a chronic prophylaxis.

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