B R I E F   C O M M U N I C A T I O N

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 A_2 (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-acetyl]aspirin (specific activity 200 mCi/mmole), was prepared by the method of Roth and Majerus (11). ^{3}H-Toluene was purchased from Amersham Radiochemicals. [^{14}C-carboxyl]aspirin (specific activity 33 mCi/mmole) was purchased from New England Nuclear. Aminohexyl Sepharose 4B was purchased from Pharmacia.

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 or 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 ^{3}H-toluene as an internal standard.

Isoelectric focusing was carried out using the method of Baumann 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'diallyl-tartardiamide 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 [^{3}H-acetyl]aspirin results in one major peak of radioactivity following SDS-PAGE (Fig. 1). Similar incubations of plasma with [^{14}C-carboxyl]aspirin yield no radiolabeled proteins following SDS-PAGE and indicate that the [^{3}H-acetyl]aspirin is acetylating a protein or proteins
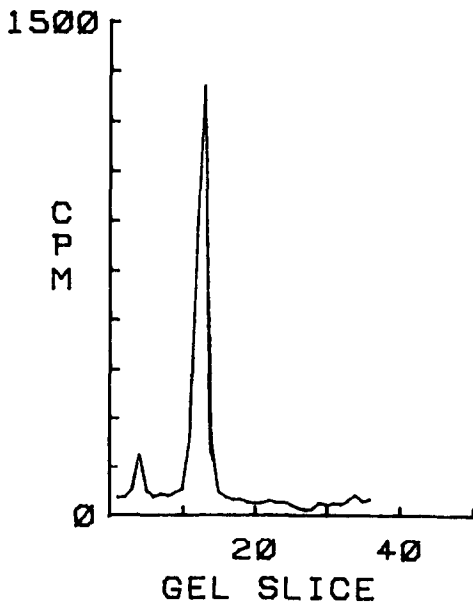
FIGURE 1

Tritium incorporation into plasma protein following incubation with 100 µ M 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 radio-
activity 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 represen-
ting albumin dimers, as only one acetylated protein was present on the iso-
electric 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
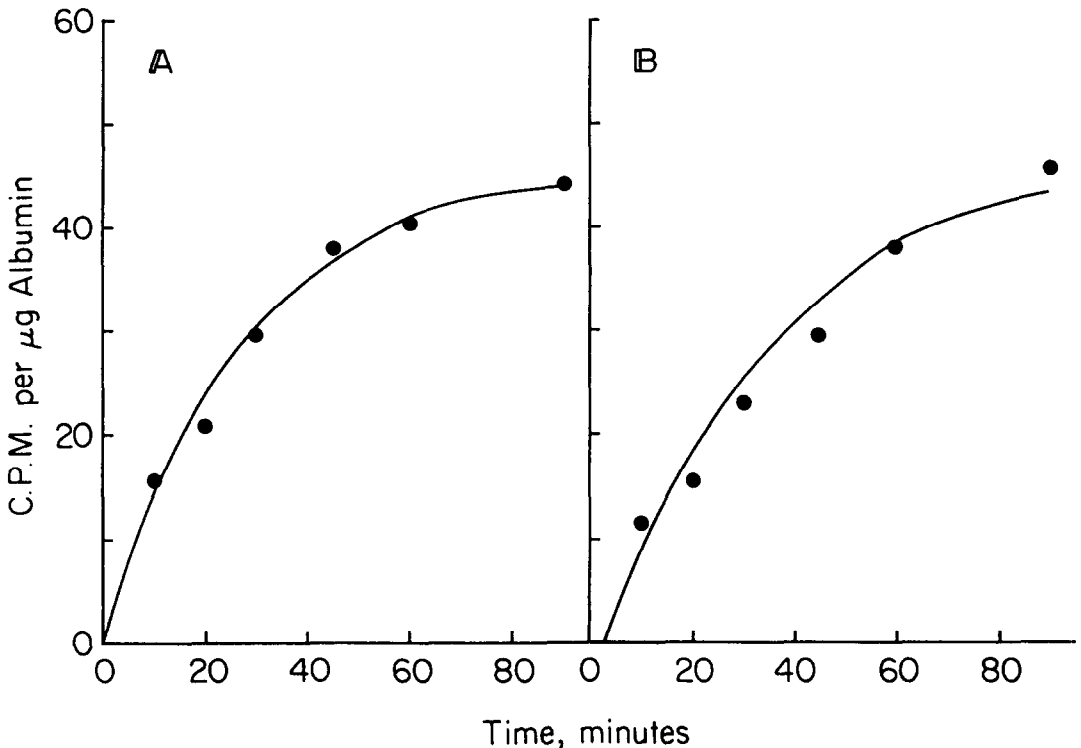conform to that expected of a second order irreversible reaction. This may be

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 contain-
ing 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 [^{3}H-acetyl]aspirin 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 in-
hibits tritium uptake in plasma by 94% (Fig. 3). The concurrent presence of
100 µM indomethacin causes a 96% inhibition of the acetylation of dilute
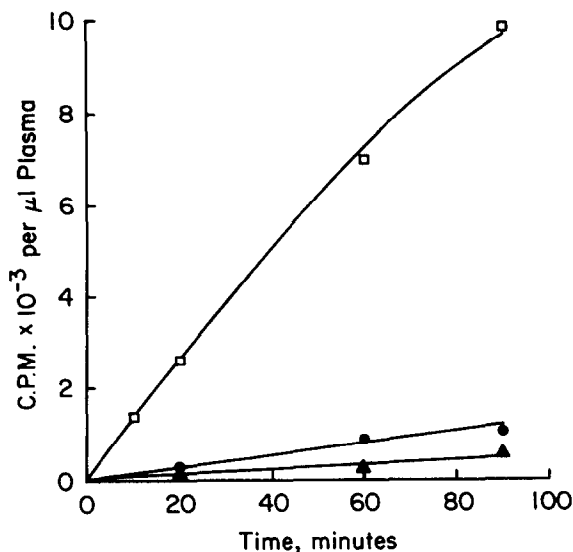plasma by 100 µM aspirin (Fig. 3).

FIGURE 3

Inhibition of tritium incor-
poration 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: radio-
active aspirin plus 100 µM
indomethacin.

DISCUSSION

Following ingestion of 650 mg aspirin, plasma aspirin levels peak at approx-
imately 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 macro-
molecules 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 A_2 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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