The Influence of Albumin and Calcium on Human Platelet Arachidonic Acid Metabolism

By Marie J. Stuart, Jonathan M. Gerrard, and James G. White

The effects of in vitro changes in calcium and albumin on human platelet arachidonic acid metabolism were evaluated. Hypoalbuminemia enhanced the conversion of released 14C-arachidonic acid from prelabeled platelet phospholipids to the metabolites of the platelet cyclooxygenase and lipoxygenase pathways. This effect was, however, associated with a decreased release of arachidonic acid in the presence of hypoalbuminemia, such that the overall conversion of released 14C-arachidonic acid to platelet thromboxane B2 was similar in the presence of physiologic albumin concentrations (3.5 g/dl) or at decreased albumin concentrations of 0.7 and 0.0 g/dl. External calcium was shown to be important for optimal platelet arachidonic acid release, with maximal release occurring at 1 mM calcium.

METABOLISM of arachidonic acid by platelets results in formation of thromboxane A2, a proaggregatory substance, and by vessel walls in the generation of the antiaggregatory hormone, prostacyclin (PGI2). It is now evident that in certain disorders accompanied by a high frequency of occlusive vascular disease, i.e., diabetes mellitus and type II (a) hyperlipidemia, an imbalance favoring the production of thromboxane and atherosclerotic vascular disease has been demonstrated, although platelet arachidonic acid metabolism and thromboxane A2 production has not been evaluated. Most recently, Yoshida has demonstrated that platelet hyperaggregability in one patient with the nephrotic syndrome was reversed by the in vitro addition of albumin to his platelet-rich plasma. Albumin also inhibits platelet aggregation and the release of radioactivity from 14C-arachidonic acid prelabeled platelets. We have extended these observations further by evaluating the effects of varying albumin concentration on the release of arachidonic acid and its subsequent conversion to products of both the cyclooxygenase and lipoxygenase pathways in platelets stimulated by thrombin. In addition, the effect of in vitro changes in calcium on platelet arachidonic acid metabolism has been evaluated.

Preparation of Platelet-Rich Plasma

Blood donors were healthy normal controls, who had not ingested any medication for at least 10 days prior to blood donation. Venous blood was collected, after informed consent, into plastic syringes and anticoagulated by using 9 volumes of blood with 1 volume citrate-phosphate-dextrose (bisodium citrate, 0.0894 M; citric acid, 0.0156 M; monobasic sodium phosphate, 0.016 M; dextrose 0.1418 M). Platelet-rich plasma (PRP) was obtained by centrifugation of the samples at 200 g for 20 min. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood for 15 min at 1800 g. Platelet counts were performed as previously described.

Evaluations of the Effects of Albumin on 14C-Arachidonic Acid Release and Metabolite Formation

Within 40 min of collection, PRP was adjusted to a platelet count of 300,000–400,000/ml with PPP. 14C-arachidonic acid (#661-New England Nuclear, specific activity 56.5 mCi/mmmole) was incubated with PRP for 90 min at 37°C. Following incubation, free arachidonic acid was removed by washing the platelets. The cells were pelleted at 4°C in the presence of 1% EDTA and resuspended in Hanks buffered salt solution (HBSS) containing 3.5% albumin (calcium and magnesium free). The procedure was repeated and the platelets finally resuspended in HBSS containing varying albumin concentrations. Human albumin, >98% pure, Cohn fraction V, fatty acid-free (<0.1 mole fatty acid/mole of albumin, Miles Laboratories, Elkart, Ind.) was used for resuspension with a final calcium concentration of 1.0 mM. The final concentration of albumin used for the washed platelet suspensions was 0.0 g/dl, 0.7 g/dl, and 3.5 g/dl. Thrombin (5.0 U/ml) was used as the activating agent and each set of experiments was run with a buffer control.

A modification of the procedure of Bills and associates was used to evaluate the thrombin-stimulated release of 14C-arachidonic acid from the prelabeled platelet phospholipids. After stirring for 5 min following addition of thrombin, CaCl2, or buffer, the samples were extracted with chloroform/methanol (1:2) and then 4.8 ml chloroform and 4.8 ml 0.1 M EDTA were added. After mixing for 30 min, the samples were centrifuged to separate the phases. The aqueous phase was removed, acidified with 1 N HCl to a pH of 3.3, and reextracted with 20 ml of chloroform methanol (5:1). The organic phases were combined and the combined extracts were evaporated under a stream of nitrogen at 37°C. Recoveries were evaluated for arachidonic acid, HHT, HETE, and thromboxane B2 at each concentration of albumin. All recoveries were greater than 97%, except the recovery of arachidonic acid in 3.5% albumin (87%) and the recoveries of thromboxane B2, which were 84% with 0% albumin.

MATERIALS AND METHODS

Preparation of Platelet-Rich Plasma

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MATERIALS AND METHODS

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min, 83% with 0.7% albumin, and 72% with 3.5% albumin. Following extraction, the organic phase was dried under vacuum, and the phospholipids separated from the free arachidonic acid and metabolites using silicic acid column chromatography, with elution profiles as previously described. The release of 4C-arachidonic acid from the platelet phospholipids was calculated by determining the percent of total radioactivity in fractions I and II containing free arachidonic acid and metabolites, and subtracting the values obtained when buffer alone was added to the platelets. Fractions I and II were then pooled, dried under vacuum, redisolved in ether, and the conversion of released 4C-arachidonic acid to thromboxane B2 evaluated by thin-layer chromatography of the free acids on silica gel G using diethyl ether:methanol:acetic acid (135:5:3 v/v) as the eluting solvent. The plates were then scanned on a thin-layer radiochromatogram scanner, and the silica gel corresponding to the thromboxane B2 (TxB2) peak counted in the scintillation counter. Further extraction of the remaining metabolites from the silica gel plates was performed, the sample being redisolved in 2 ml ether. The arachidonic acid and remaining metabolites were then methylated, using diazomethane, and separated on a thin-layer chromatogram (silica gel B), using a solvent system containing the organic layer of 100:100:50 isooctane:water:ethyl acetate. The thin-layer plates were scanned using a Bershov radiochromatogram scanner, and the silica gel corresponding to the thromboxane B2 peak counted in the scintillation counter. Results were expressed as the mean of from 8 to 10 experiments. Phospholipids separated from the free arachidonic acid and metabolites evaluated following methylation using diazomethane. Separation of metabolites was performed on a thin-layer chromatogram (silica gel G) using a solvent system containing the organic layer of 100:100:50 isooctane:water:ethyl acetate. The thin-layer plates were scanned on a thin-layer radiochromatogram scanner, and the conversion of arachidonic acid to product quantitated as previously described. Results were expressed as the mean of 6-7 experiments.

RESULTS

Effect of Albumin on the Thrombin-Induced Release of 4C-Arachidonic Acid From Platelets and Its Conversion to Prostaglandin Metabolites

As depicted in Fig. 1, the addition of thrombin (5.0 U/ml) to platelets caused the variable release of 4C-arachidonic acid depending on the albumin concentration in the external platelet milieu. Release was greatest when the albumin concentration was 3.5 g/dl (32.2% ± 1.7%). This value was increased significantly (p < 0.001) when compared to a value of 28.2% ± 2.1% obtained at an albumin concentration of 0.7 g/dl. In the absence of external albumin, the release of arachidonic acid from prelabeled platelet phospholipids was 18.3% ± 1.1%. This value was adjusted to final platelet concentration of 5 × 10^8 platelets. The suspension was divided into 4 equal aliquots to which was added either (1) thrombin (5 U/ml) and CaCl2 (4.0 mM final concentration); (2) thrombin (5 U/ml) and CaCl2 (1.0 mM); (3) thrombin (5 U/ml) without CaCl2; or (4) buffer control and CaCl2 (4.0 mM).

Following extraction, silicic acid column chromatography, and evaluation of the 4C-arachidonic acid release from the prelabeled platelet phospholipids, fractions I and II were pooled, dried under vacuum, dissolved in ether, and the conversion of released 4C-arachidonic acid to metabolites evaluated following methylation using diazomethane. Further extraction of the remaining metabolites from the silica gel was performed, the sample being redisolved in 2 ml ether. The arachidonic acid and remaining metabolites were then methylated, using diazomethane, and separated on a thin-layer chromatogram (silica gel B), using a solvent system containing the organic layer of 100:100:50 isooctane:water:ethyl acetate. The thin-layer plates were scanned on a thin-layer radiochromatogram scanner, and the conversion of arachidonic acid to product quantitated as previously described. Results were expressed as the mean of 6-7 experiments.

Effect of Albumin on the Thrombin Stimulated Arachidonic Acid Release from Platelets

Evaluation of the Effects of Calcium on 4C-Arachidonic Acid Release and Subsequent Metabolism by the Platelet

Similar experiments were performed where 4C-arachidonic acid (specific activity 36.5 mCi/mmmole) was incubated with PRP for 90 min at 37°C. Following incubation, free arachidonic acid was removed by washing the platelets. The cells were pelleted at 4°C in the presence of 1% EDTA and resuspended in HBSS containing 3.5% albumin (calcium and magnesium free). The procedure was repeated, and the platelets finally resuspended in HBSS containing 3.5% albumin and a final concentration of 1 mM MgCl2 and

![Fig. 1](https://www.bloodjournal.org/)

**Fig. 1.** Effect of albumin on the thrombin (5.0 U/ml) stimulated release of 4C-arachidonic acid from prelabeled platelet phospholipids. Results expressed as the mean ± 1 SE.
Effect of Albumin on the Percent of Total Platelet [14C] Arachidonic Acid That Was Converted to Thromboxane B2

Fig. 2. Effect of albumin on the conversion by platelets of thrombin (5.0 U/ml) released [14C] arachidonic acid to the prostaglandin metabolites thromboxane B2, HHT, and HETE. Results expressed as the mean ± 1 SE.

![Graph showing the effect of albumin concentration on the conversion of arachidonic acid to thromboxane B2.](image)

Effect of Albumin on the Percent of Total Platelet [14C] Arachidonic Acid That Was Converted to Thromboxane B2

Fig. 3. Effect of albumin on the percent of total platelet arachidonic acid that was converted to thromboxane B2 by human platelets in the presence of thrombin (5.0 U/ml). Results expressed as the mean ± 1 SE.

![Graph showing the effect of albumin concentration on the percent conversion of arachidonic acid to thromboxane B2.](image)

Effect of Calcium on Thrombin Stimulated Arachidonic Acid Release from Platelets

Addition of thrombin (5.0 U/ml) to platelets caused variable release of [14C] arachidonic acid from the prelabeled phospholipids depending on the external calcium concentration, as depicted in Fig. 4. Release was greatest when the calcium concentration was 1 mM (32.2% ± 2.4%; 1 SE). This value was increased (p < 0.01) when compared to the values of 28.0% ± 1.5% and 26.9% ± 2.4% obtained for arachidonic acid release when calcium concentrations were 4 mM and 0 mM, respectively. Released arachidonic acid was converted to 12L-hydroxy-5,8,10,14 eicosatetraenoic acid or HETE, 12L-hydroxy-5,8,10 heptadecatrienoic acid or HHT, and thromboxane B2 or TXB2. As seen in
PLATELET ARACHIDONIC ACID METABOLISM

Effect of Calcium on Thrombin Stimulated Conversion to Metabolites (TXB2, HHT and HETE) of Arachidonic Acid Released from Platelets

Fig. 5. Effect of calcium on the conversion by platelets of thrombin (5.0 U/ml) released 14C-arachidonic acid to the prostaglandin metabolites thromboxane B2, HHT, and HETE. Results expressed as the mean ± 1 SE.

Fig. 5, conversion of the released arachidonic acid to metabolites was greatest at calcium concentrations of 4 and 1 mM (49.3% ± 3.7% and 47.6% ± 2.7%), respectively. These values were increased (p < 0.01) when compared to a value of 36.5% ± 2.9% obtained in the absence of externally added calcium.

DISCUSSION

The present study demonstrates that albumin has differing effects on the various stages of platelet arachidonic acid metabolism. The formation of the various end-products of platelet arachidonic acid metabolism results from two steps: the release of precursor fatty acid from cell phospholipids, and its subsequent conversion by either one of two pathways, the lipoxygenase or cyclooxygenase. Platelet lipoxygenase converts arachidonic acid to HPETE (12-l-hydroxy-5,8,10,14-eicosatetraenoic acid), which is further reduced to the final proaggregatory metabolite, is formed enzymatically from either of these cyclic endoperoxides. However, it is unstable, undergoing rapid hydrolysis to thromboxane B2, which is stable and can be used as a measure of the amount of thromboxane A2 produced during cell activation. A major alternative pathway for metabolism of PGG2 and PGH2 is by further degradation to the hydroxy fatty acid HHT and malonyldialdehyde. In addition to these major pathways, small amounts of PGG2 and PGH2, are converted to PGE2, PGD2, and PGF2α.

From our results, it appears that albumin has a dose-dependent effect on the thrombin-induced release of 14C-arachidonic acid from prelabeled platelet phospholipids, with increased release occurring at an albumin level of 3.5 g/dl, when compared to a hypoalbuminemic level (0.7 g/dl), or in its total absence. However, evaluation of the subsequent conversion of released arachidonic acid to metabolites of the platelet lipoxygenase and cyclooxygenase pathways revealed an opposite effect. Conversion to HHT, HETE, and TXB2 was markedly increased in the absence of albumin (96.1%). Increasing the albumin concentration decreased the percent conversion to metabolites, with 53.7% conversion in the presence of 0.7 g/dl albumin, and an even lower value of 41.7% conversion at a physiologic albumin level of 3.5 g/dl. Evaluation of proaggregatory thromboxane A2 by assaying the levels of its stable end-product, thromboxane B2, revealed a marked increase in B2 formation in the absence of albumin. When the percent of total platelet 14C-arachidonic acid that was converted to thromboxane B2 was evaluated, however, no significant differences were observed (Fig. 3) in the presence of varying albumin concentrations. Thus, the differing effects of albumin on arachidonic acid release and subsequent metabolism negated each other.

Previous work by Bills and coworkers had demonstrated that the appearance of oxygenated products of arachidonic acid was reduced in the presence of albumin. However, in contrast to our finding of an increase in the release of 14C-arachidonic acid in the presence of increasing albumin concentrations, these authors showed that albumin inhibited the release of arachidonic acid from prelabeled phospholipids. Most recently, Deykin has demonstrated an optimal effect of the addition of 0.05 g/dl delipidated albumin on the release of arachidonic acid from platelet phospholipid. Increasing the albumin concentration to 0.5 g/dl did not enhance arachidonic acid release. Our studies show that by increasing the albumin concentration still further (to 0.7 and 3.5 g/dl) further enhancement of arachidonic acid release occurred. Differences in the concentrations of albumin used to maximally facilitate arachidonic acid release may be attributable to differing fatty acid content of the fatty acid "free" albumin preparations. Other studies on the effect of albumin on platelet arachidonic acid metabolism have been performed by Yoshida and associates, who have...
demonstrated that, in the presence of albumin, there was an increase in the amount of arachidonic acid bound to albumin following platelet stimulation and a decrease in conversion to prostaglandin metabolites, as confirmed by our studies. However, unlike Yoshida, we did not find a biphasic response with maximum release of arachidonic acid from prelabeled phospholipids occurring at an albumin concentration of 0.7 g/dl. In contrast, in our studies maximum release was observed at physiologic levels of albumin with decreasing levels of release as the albumin concentration was successively lowered.

In light of our data, the inhibitory effects of albumin on collagen, ADP, and arachidonic acid induced aggregation in vitro, as shown by various workers, does not appear to be due to an absolute reduction in the formation of the proaggregatory thromboxane A2. Other causes for platelet hyperaggregability in the nephrotic syndrome warrant investigation. It is recognized that cholesterol loading of platelet membranes causes an increase in the sensitivity of platelets to various aggregating agents and increases serotonin release. Therefore, the hypercholesterolemia seen in the nephrotic syndrome may play a role in the platelet hyperfunction seen in this disorder. Since the hypoalbuminemia seen in the nephrotic syndrome is associated with a decrease in total serum calcium, the effect of external calcium on arachidonic acid metabolism was also assessed.

Previous studies by Rittenhouse-Simmons and associates had demonstrated an increase in the thrombin-induced release of arachidonic acid from platelet phospholipids in the presence of external calcium (4 mM) when compared to thrombin activation in the presence of 1 mM EGTA. Our results confirm her findings and also demonstrate that the optimal concentration for thrombin-induced release of arachidonic acid appears to be at an external calcium concentration of 1 mM. Deykin and coworkers have recently demonstrated that in the presence of calcium, the formation of HETE (lipoxygenase pathway) was significantly increased when compared to either TxB2 or HHT (cyclooxygenase pathway). Although an increase in the total products formed (HETE, HHT, and TxB2) was observed at lower calcium concentrations may relate to the findings of Mustard et al., showing that platelet aggregation and secretion is much more pronounced in citrate platelet-rich plasma. This effect was found to be related to the lower calcium concentration caused by citrate. Enhanced release of arachidonic acid at the low calcium concentration (1 mM) could explain the enhanced second wave of aggregation and secretion, processes in which release of arachidonic acid and synthesis of prostaglandins appear to be critical.

In conclusion, using fatty-acid-free albumin in an in vitro system, we have shown that lowering the albumin concentration does not influence the percent of total platelet 14C-arachidonic acid converted to thromboxane B2 by the human platelet. Hypoalbuminemia enhances the conversion of released arachidonic acid to metabolites of the platelet lipoxygenase and cyclooxygenase pathways. However, this effect is balanced by a decrease in the release of arachidonic acid from prelabeled platelet phospholipids in the presence of decreased albumin levels. External calcium is important for optimal platelet arachidonic acid release, with maximal release occurring at 1 mM calcium. This finding may explain previous observations that platelet aggregation and secretion is most pronounced in citrated platelet-rich plasma.

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