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 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.

MATERIALS AND METHODS

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 200,000–400,000/ml with PPP. 14C-arachidonic acid (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 cells 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 associates14 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.

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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.15 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, redissolved 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 redissolved 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 then scanned using a Berthrold radiochromatogram scanner, and the silica gel corresponding to the thromboxane B² (TxB²) peak counted in the scintillation counter.

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

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.

decreased \( (p < 0.01) \) when compared to either of the values obtained in the presence of albumin. As shown in Fig. 2, in the presence of 3.5 g/dl of albumin, 41.7% ± 2.1% of the released arachidonic acid was converted to total metabolites (HETE, HHT, and TxB₂). This value was less \( (p < 0.01) \) than the percent conversion when the albumin concentration was reduced to 0.7 g/dl (53.7% ± 1.2%). In the absence of external albumin, conversion to metabolites was markedly increased \( (p < 0.001) \) to 96.1% ± 1.1%. Similarly, the percent of TxB₂ formed was greatest \( (p < 0.01) \) in the absence of albumin (19.7% ± 2.3%) when compared to mean values of 11.2% ± 1.3% and 12.8% ± 1.9% in the presence of 3.5 and 0.7 g/dl albumin, respectively. However, evaluation of the overall conversion of [14C]-arachidonic acid to TxB₂ in the absence or presence of varying albumin concentrations were similar. As depicted in Fig. 3, values of 3.8% ± 0.6%, 3.7% ± 0.7%, and 3.7% ± 0.4% were obtained at albumin concentrations of 3.5, 0.7, and 0.0 g/dl, respectively.

Effect of Calcium on the Thrombin-Induced Release of [14C]-Arachidonic Acid From Platelets and Its Conversion to Prostaglandin Metabolites

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 12-L-hydroxy-5,8,10,14 eicosatetraenoic acid or HETE, 12-L-hydroxy-5,8,10 heptadecatrienoic acid or HHT, and thromboxane B₂ or TxB₂. As seen in
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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.

...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 PGE₁, 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.

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-hydroxy-5,8,10,14-eicosatetraenoic acid), which is further reduced to the final product HETE (12l-hydroxy-5,8,10,14-eicosatetraenoic acid). Fatty acid cyclooxygenase converts arachidonic acid to prostaglandins and their metabolites. The first recognizable intermediates are the cyclic endoperoxides PGG₂ and PGH₂. Thromboxane A₂, the most potent 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.
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 A$_2$. 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 TXB$_2$ or HHT (cyclooxygenase pathway). Although an increase in the total products formed (HETE, HHT, and TXB$_2$) was seen in the presence of external calcium (1 and 4 mM), no separation of HHT from HETE was performed in our study. The increased release of arachidonic acid at low 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 $^{14}$C-arachidonic acid converted to thromboxane B$_2$ 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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