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Increased sensitivity of arachidonic acid-induced platelet aggregation in the presence of carbon dioxide

P.J. Kerry & Catherine J. Paton

Division of Blood Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB

1 The effects of carbon dioxide on citrated human platelet-rich plasma (PRP) have been studied as a means of imitating the changes in pH and P_{CO_2} observed in inflammation and tissue fluid stasis.

2 Adenosine diphosphate (ADP)-induced platelet aggregation was inhibited in CO_2-treated PRP.

3 In contrast, CO_2-treated platelets were rendered up to eight times more sensitive to sodium arachidonate and this effect could be imitated by the addition of exogenous calcium 1 min before the addition of arachidonate.

4 The effects of CO_2 on ADP-induced and arachidonate-induced aggregation were abolished if the CO_2 was allowed to disperse from treated PRP subsequently exposed to air, suggesting no permanent alteration in platelet metabolism.

5 The increased sensitivity of arachidonate-induced aggregation with lowered pH may be a significant factor in influencing platelet behaviour in haemostasis.

Introduction

The effect of pH on adenosine diphosphate (ADP)-induced platelet aggregation has been studied with particular consideration to the handling and storage of platelet concentrates intended for infusion (Han & Ardlie, 1974; Patscheke, 1981; Watts, Tunbridge & Lloyd, 1983) and this work supports the suggestion that acid conditions are beneficial to the viability of fresh and stored human platelets (Morrison & Baldini, 1967).

There is evidence that tissue fluids become more acid during inflammation and Schade (1924), for example, observed that pH fell to 5.6 during abscess formation. P_{CO_2} and P_{O_2} measurements in wound fluid from an inflammatory lesion in sheep suggested a degree of stasis (Greenwood & Kerry, 1975) and the pH of this sterile exudate has been measured as low as 6.9 (Greenwood, personal communication).

Rogers (1972) developed the simple procedure of layering carbon dioxide enriched air over samples of platelet-rich plasma to control pH *in vitro* and this technique was used to demonstrate that ADP- and adrenaline-induced platelet aggregation was inhibited in CO_2-treated platelets. Rogers postulated that these results might have some clinical relevance in acute acid-base disorders of respiratory origin. Experiments in which P_{CO_2} was kept constant but in which pH was allowed to vary showed that pH, not P_{CO_2}, was important in determining the sensitivity of human platelet aggregation to ADP and adrenaline (Lamberth, Warriner & Batchelor, 1974).

The effects of carbon dioxide on human platelet aggregation *in vitro*, with the concomitant changes in pH, have been studied as a means of imitating the changes in pH and P_{CO_2} observed in inflammation and tissue fluid stasis. A preliminary account of this work has been presented to the British Pharmacological Society (Kerry & Paton, 1983).

Methods

Preparation of platelet-rich plasma (PRP)

Whole blood from healthy, aspirin-free human volunteers was collected into plastic tubes containing 1 part 3.8% w/v tri-sodium citrate to 9 parts blood. PRP was prepared by centrifugation at 600 *g* for 10 min. The platelet suspension was stored at room temperature and all experiments were completed within 4 h of venepuncture.

Carbon dioxide-treated platelets

PRP (4 ml) was transferred to a new polystyrene test tube (10 ml, 14 mm i.d.) and a stream of CO_2 at room temperature was directed on to the surface of the platelet suspension. A volume of untreated PRP was retained for control purposes. The pH of CO_2-treated and control platelets was monitored at regular intervals with a foetal blood pH analyzer (System RM1306, Radiometer). After exposure to CO_2 for approximately 40 min, a stable pH of 6.3 was achieved; at this stage the stream of CO_2 was discontinued and the treated PRP allowed to equilibrate with air. The pH was again monitored at frequent intervals and samples taken for aggregation studies as the pH increased.

Platelet aggregation

Platelet aggregation was measured at 37°C in a Payton single channel aggregometer, with a stirring speed of 1,100 r.p.m. PRP (200 µl) was incubated in disposable polystyrene Durham tubes for 2 min in the aggregometer before addition of aggregating agents. Changes in light transmission were recorded for at least 5 min.

Reagents

Arachidonic acid (grade I) and ADP were obtained from Sigma London Chemical Company. Arachidonic acid was prepared as the sodium salt and stored as an ethanolic solution sealed under nitrogen.

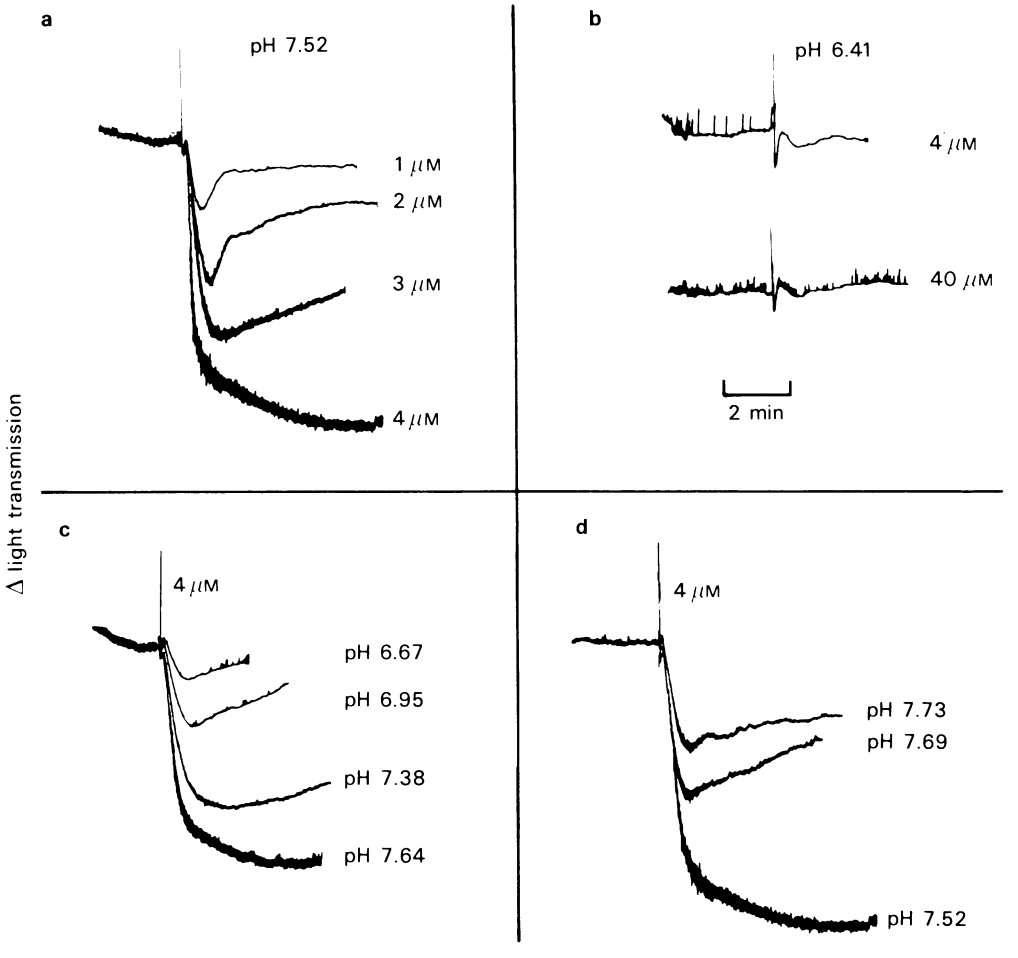

Figure 1 Effect of carbon dioxide on ADP-induced platelet aggregation from a typical experiment. The recordings have been superimposed for each part of the experiment and final concentrations of reagents are given. (a) Dose-responses of control citrated PRP to ADP. (b) Inhibitory effect of CO_2 on ADP-induced aggregation in citrated PRP. (c) Reversal of CO_2 influence on ADP-induced platelet aggregation, with treated PRP in equilibrium with air. (d) Deterioration of control PRP response to ADP as pH increased with time.

Just before use, the ethanol was removed by evaporation and the sodium arachidonate redissolved in distilled water to give a stock solution of 10 mg ml^{-1}. An aqueous stock solution ADP (2.0 mM) was stored in 1.0 ml aliquots at − 20°C and a new ampoule, kept on ice, was used for each experiment.

Results

ADP-induced aggregation

The results from one representative experiment are illustrated in Figure 1. The dose-responses of control PRP, pH 7.52, to ADP show graded responses with reversible aggregation to 1.0 μM and full aggregation to 4.0 μM ADP (Figure 1a). ADP 4.0 μM and ADP concentrations up to 40 μM failed to elicit a response other than a transient shape change in CO_2-treated PRP, pH 6.41 (Figure 1b). However, the inhibitory effect of CO_2 on aggregation in response to 4.0 μM ADP was gradually lost as the CO_2 dispersed on exposure of CO_2-treated PRP to air; full aggregation was obtained at a pH value between 7.38 and 7.64 (Figure 1c). During the course of the experiment, the pH of control PRP continuously in equilibrium with air increased from an initial pH 7.52 in freshly prepared PRP ('0' time) to pH 7.69 three hours and pH 7.73 four hours after preparation of PRP. As the pH increased so the sensitivity of PRP to ADP decreased (Figure 1d).

Arachidonic acid-induced aggregation

Figure 2a illustrates the dose-responses of control PRP, pH 7.46 to sodium arachidonate. There was an increase in the shape change response with increasing doses of 0.2 mM to 0.8 mM sodium arachidonate but full aggregation was only observed at an arachidonate concentration of 1.6 mM. Carbon dioxide-treated PRP, pH 6.51, gave full aggregation in response to 0.4 mM arachidonate, although the onset of aggregation was slightly delayed and the shape change more pronounced in comparison with the control PRP response to 1.6 mM arachidonate (an approximately four fold increase in sensitivity; Figure 2b). The sensitivity to 0.4 mM arachidonate was lost as the treated PRP released CO_2 on exposure to

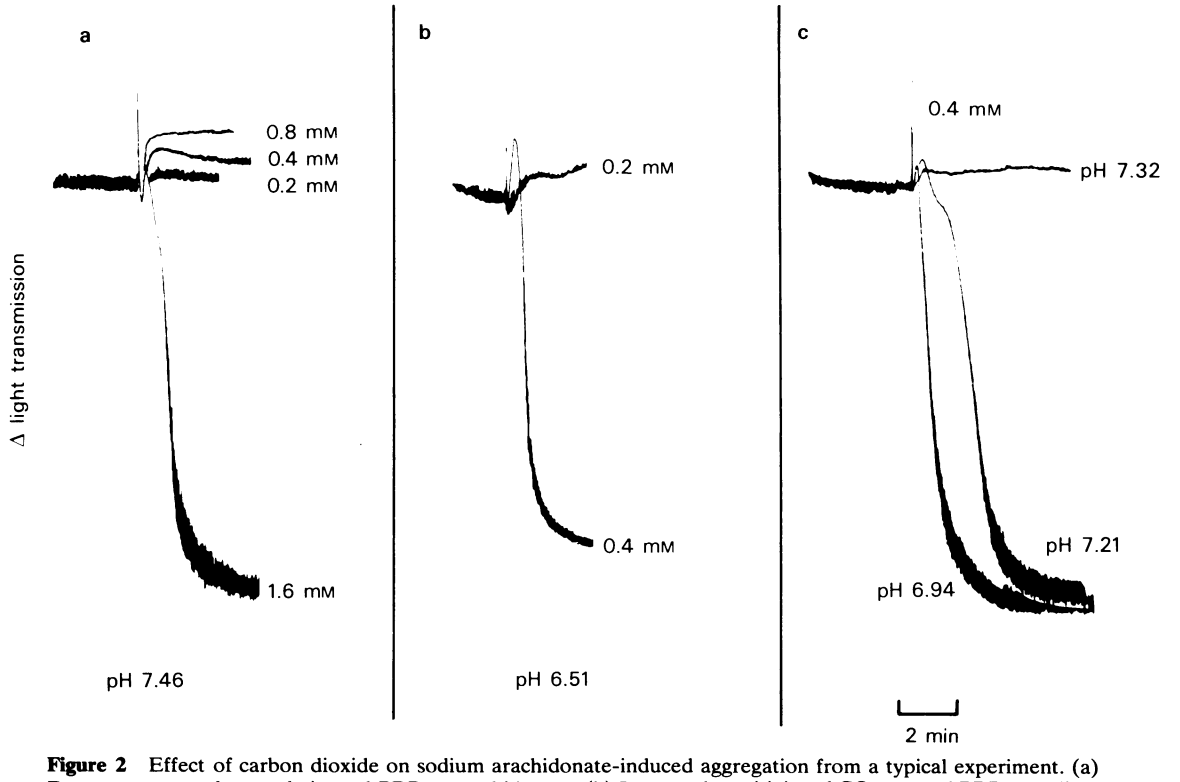

Figure 2 Effect of carbon dioxide on sodium arachidonate-induced aggregation from a typical experiment. (a) Dose-responses of control citrated PRP to arachidonate. (b) Increased sensitivity of CO_2-treated PRP to sodium arachidonate. (c) Reversal of CO_2 influence on arachidonate-induced platelet aggregation, with treated PRP in equilibrium with air. Note the sudden loss of response of PRP to 0.4 mM arachidonate between pH 7.21 and pH 7.32.

air; this loss of sensitivity was observed in the narrow pH range 7.21–7.32 (Figure 2c).

These results are representative of those from seven experiments but PRP from two other individuals (not known to have taken non-steroidal anti-inflammatory drugs) was less sensitive to sodium arachidonate, requiring a concentration of 3 mM to give full aggregation (about double the concentration of arachidonic acid needed to give full platelet aggregation in the other subjects). However, CO_2-treated platelets from these two volunteers did aggregate to the same dose of arachidonate (0.4 mM) that gave full aggregation in CO_2-treated PRP from the other donors. Carbon dioxide-treated PRP from these two individuals therefore showed an approximate eight fold increase in sensitivity to arachidonate when compared with their respective control (untreated) PRP.

The effects of pH changes on arachidonate-induced aggregation in CO_2-treated PRP could be imitated by the addition of calcium in amounts less than that necessary to saturate the chelating capacity of citrate anticoagulant (Figure 3). In this typical experiment, control PRP, pH 7.55, did not aggregate in the presence of 0.8 mM arachidonate but gave full aggregation when the concentration was 1.6 mM (Figure 3a). With CO_2-treated PRP, pH 6.34, full aggregation was obtained with 0.8 mM arachidonate (Figure 3b). In Figure 3c, various amounts of 1.0 M calcium chloride solution were added to PRP, pH 7.55, 1 min before the addition of 0.8 mM arachidonate. This same concentration of arachidonic acid in the presence of 2.5 mM exogenous calcium chloride produced a small platelet shape change but no aggregation and, in the presence of 5.0 mM exogenous $CaCl_2$, gave a full but delayed aggregation. Rapid full aggregation was achieved with 0.8 mM arachidonate in PRP containing 10.0 mM exogenous $CaCl_2$. This latter sample of PRP clotted 5 min after the addition of calcium. Other experiments indicated that calcium, in concentrations necessary to enhance the sensitivity of arachidonate-induced aggregation, did not cause aggregation on its own within the usual time course of each aggregation (approximately 5 min). Platelet aggregation and plasma clotting did occur 10–15 min after the addition of 10.0 mM $CaCl_2$ in the absence of arachidonic acid.

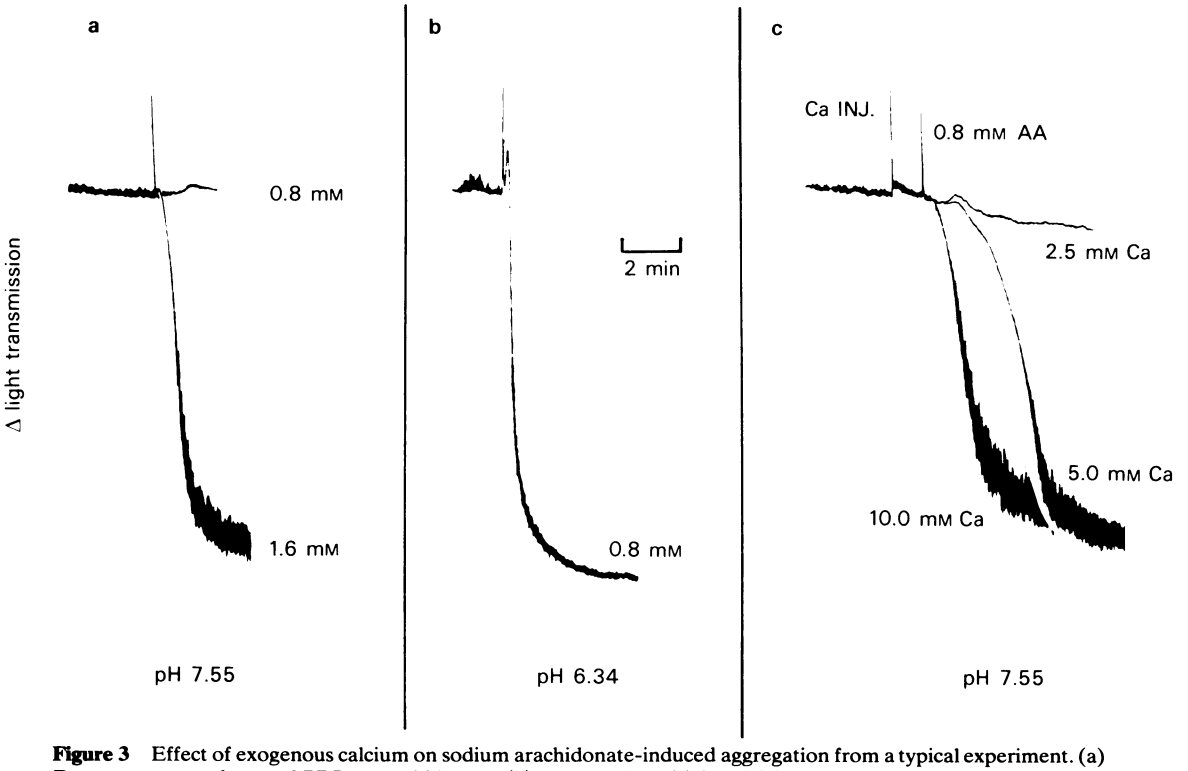

Figure 3 Effect of exogenous calcium on sodium arachidonate-induced aggregation from a typical experiment. (a) Dose-responses of control PRP to arachidonate. (b) Increased sensitivity of CO_2-treated PRP to sodium arachidonate. (c) Response of PRP to 0.8 mM arachidonate in the presence of three different concentrations of exogenous $CaCl_2$ (added to PRP 1 min before arachidonate).

Discussion

In these experiments the effects of carbon dioxide on human platelet aggregation have been examined with particular reference to two agonists, arachidonic acid and ADP. Our experiments with ADP essentially confirm the findings of Rogers (1972), namely that CO_2 (or CO_2-enriched air) inhibits the response of citrated PRP to a concentration of ADP giving full aggregation with control PRP. This inhibition could not be overridden by a ten fold increase in ADP but platelet responsiveness was gradually restored as the PRP was allowed to equilibrate with air. It is still unclear how lowered pH inhibits ADP aggregation but it has been suggested that pH dependent phenomena such as net surface charge and platelet cyclic AMP metabolism may be involved (Rogers 1972). Other experiments in this laboratory indicate that ADP by itself is stable under the acid conditions encountered in CO_2-treated PRP.

In contrast, the sensitivity of sodium arachidonate-induced platelet aggregation was enhanced four to eight fold in the presence of CO_2. The increase in sensitivity varied depending on the initial sensitivity of control PRP to this agonist. As with ADP, the effect could be reversed as the CO_2 was allowed to disperse on the exposure of treated PRP to air but, unlike ADP, the increase in sensitivity was lost completely within a relatively narrow pH range (pH 7.21 – pH 7.32).

The effect of CO_2 on arachidonate-induced platelet aggregation is almost certainly complex and several possible mechanisms, singly or in various combinations, can be invoked in an attempt to explain the phenomenon.

(1) The increased hydrogen ion concentration observed in PRP exposed to CO_2 may displace calcium from citrate anticoagulants. Our own experiments in which added calcium imitated the effects of CO_2 on arachidonate-induced aggregation lend support to the displacement of calcium from citrate with lowered pH. Other workers have shown that, with a decrease in hydrogen ion concentration, the calcium-citrate complex dissociates less readily (Charberek & Martell, 1959) and therefore lowering pH will allow the calcium-citrate complex to dissociate more easily, making available supplies of free calcium for platelet aggregation.

Calcium in the blood is at least partly bound to plasma proteins (principally albumin but also globulin) and the extent of binding is governed by the chemical nature of the protein, temperature, pH and, in all probability, other as yet poorly understood factors. The effect of pH is particularly important; albumin binds more calcium under alkaline conditions and less calcium at acid pH (Myers, 1962; Pedersen, 1972). It is therefore possible that more free calcium is available in CO_2-treated PRP and this may also account for the increased sensitivity of platelet aggregation to arachidonic acid.

(2) Increased hydrogen ion concentration could also influence substrate availability by displacing arachidonic acid from protein bound stores. Although there is much information on the binding of fatty acids to albumin (Spector, 1975), little is known of the effect of small physiological (and pathological) changes in pH on arachidonic acid binding to albumin. Experiments have been carried out in this laboratory to examine this possibility but as yet no satisfactory method has been found to study this hypothesis. Equilibrium dialysis would not be suitable because of the inability of fatty acids containing 12 or more carbon atoms to pass through ordinary dialysis membranes at pH 7.4 (Spector, 1975) but the equilibrium partition method may prove more satisfactory in future experiments. The situation could be further complicated due to interactions between calcium, arachidonic acid and plasma albumin, with either calcium or arachidonic acid disturbing the equilibrium between albumin and the other agent. Other experiments with washed platelets are being carried out to provide more information on the mechanisms involved.

(3) A further possibility is that increased hydrogen ion concentration may exert a direct action on platelet metabolism and/or function. There is no information from our experiments as to platelet intracellular pH, which could well differ from that of the extracellular environment in CO_2-treated (and possibly control) PRP. Changes in pH could also bring about conformational changes in prostanoid receptors and it may also be that platelet cyclooxygenase is sensitive to changes in pH.

The use of tri-sodium citrate, $3.8\,g\,l^{-1}$ final concentration, as anticoagulant has certain limitations in this experimental system, especially in view of the influence of calcium on platelet function (Detwiler, Charo & Feinman, 1978) and the calcium chelating capacity of citrate. However, by extrapolation, these very limitations might suggest a far more sensitive system *in vivo* where the citric acid concentration in whole blood from adults is about $15\,mg\,l^{-1}$ (Diem & Lentner, 1975). The chelating capacity due to citrate *in vivo* will be less than in PRP containing exogenous citrate and it therefore follows that any physiological or pathological changes in pH could displace calcium from endogenous citrate (and possibly albumin bound stores) far more readily, rendering circulating platelets more sensitive to arachidonic acid (and other agents involved in platelet activation and aggregation).

Alternative anticoagulants such as heparin could well prove useful in extending this study. However, heparin coats cellular components of blood, making

them adhere to each other more readily (Currie, 1967) and there is also some evidence that heparin itself can contribute to platelet aggregation (Eika, 1972; Salzman, Rosenberg, Smith, Lindon & Favreau, 1980).

In these experiments, changes in pH attributable to dissolved CO_2 altered the sensitivity of platelet aggregation to ADP and arachidonic acid. The increased sensitivity of arachidonate-induced aggregation with lowered pH may be a significant factor in influencing the behaviour of platelets in haemostasis.

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