PAPERS AND ORIGINALS

Antithrombotic potential of dihomo-gamma-linolenic acid in man

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Summary

The effects of orally ingested dihomo-γ-linolenic acid (DHLA), the natural biosynthetic precursor of prostaglandin E_1 (PGE_1), were assessed in human volunteers. Single doses of DHLA (0·1–2g) increased the proportion of DHLA relative to arachidonic acid in plasma and platelets and also increased the ex-vivo capacity of platelets to produce PGE_1 and PGE_2. More pronounced effects were observed during sustained treatment (five days to four weeks) when DHLA also accumulated in red cell membranes. These biochemical changes were accompanied by potentially antithrombotic changes in haemostatic function. The most common effect, which was consistently detected after 0·1-g single doses of DHLA or its methyl ester, was a decrease in plasma heparin-neutralising activity. Inhibition of platelet aggregation induced by adenosine diphosphate was also detected, though this was generally less pronounced. Sustained treatment in one subject also produced definite inhibition of ristocetin-induced platelet aggregation. There was only one possible adverse effect—a transient cough in a subject with a history of asthma.

DHLA therefore seems to have considerable potential as an agent for preventing and treating human thromboembolic disease.

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Introduction

Recent advances in the understanding of the roles of prostaglandins in platelet function have suggested new possibilities for therapeutic intervention in human thromboembolic disease. This report is concerned with one such possibility—the use of dihomo-γ-linolenic acid (DHLA), a naturally occurring polyunsaturated fatty acid which is the biosynthetic precursor of prostaglandin E_1 (PGE_1).

When platelets are appropriately stimulated, prostaglandin-precursor fatty acids are mobilised from phospholipid pools. These fatty acids are metabolised to various prostaglandins and related products, which have different modulating effects on platelet function.[1] Metabolic pathways of the precursor fatty acids DHLA and arachidonic acid (AA) are shown in fig 1. The relative amounts of DHLA and AA in platelets and other tissues are determined largely by dietary constituents and the degree to which DHLA is converted to AA. In most tissues, including platelets, there is much more AA than DHLA. Probably because of this, and because both fatty acids compete for the enzyme cyclo-oxygenase, metabolites of AA, such as the strongly proaggregatory thromboxane A_2 (TxA_2), are generated in preference to metabolites of DHLA such as PGE_1. DHLA-derived PGE_1 is one of the most potent known inhibitors of platelet function and has other properties that make it attractive as a potential antithrombotic agent: it produces vasodilatation, promotes increased red cell flexibility in vitro,[2] and inhibits the development of artificially induced arterial thrombosis in

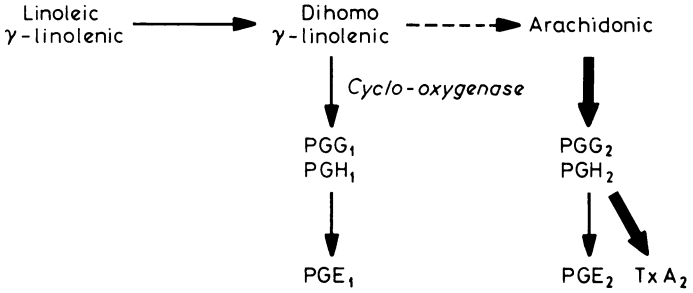

FIG 1—Some metabolic pathways of DHLA and AA.

animals.[3] PGE_1 has been tried as an antithrombotic agent in man but is unlikely to gain a useful place in treatment[4] because it is rapidly metabolised.

An alternative to administering PGE_1 itself might be to use its precursor DHLA to increase the dietary ratio of DHLA to AA and thereby change the balance of platelet prostaglandin biosynthesis towards PGE_1 and away from the proaggregatory metabolites of AA. An advantage of this approach would be the release of PGE_1 at its required site of action. A possible problem would be conversion of DHLA to AA; the extent to which this occurs in man has previously been unknown. The action and effects of DHLA have been extensively examined by Willis *et al*, who have shown that DHLA inhibits aggregation of human platelets in vitro and animal platelets when tested ex vivo after oral administration of DHLA.[1 5] Our objective was to study the effects of oral DHLA on haemostasis and on fatty acid and prostaglandin biochemistry in man.

Subjects and methods

DHLA free acid and its methyl and ethyl esters (DHLmethyl ester' DHLethyl ester) were obtained from Roche Products Ltd, Welwyn Garden City; Nuchek Prep Inc, Elysian, Minnesota; or Bio-Oils Research Ltd, Nantwich. All materials were over 99% pure at the time of ingestion and were taken sealed under nitrogen in soft gelatin capsules.

The volunteers comprised eight healthy men aged 29-47 years. All were members of the medical or scientific staff and were fully informed of the nature and possible hazards of the experiments. No medication apart from vitamin E was taken during the studies or the two weeks before.

Tests of haemostasis were generally carried out as previously described.[6] Plasma heparin-neutralising activity was measured using one or more of three different methods.[7-9] Platelet aggregation was studied using either a Born mark III aggregometer or a Payton module. Blood used for preparing platelet-rich plasma was routinely collected into 3·8% trisodium citrate (1 part citrate to 9 parts blood), although very occasionally tests were carried out with half-strength citrate and with an unadjusted platelet count. Platelet-rich plasma platelet counts were adjusted to the range 200-250 $\times 10^9$/l. Chemical and haematological values were followed using standard automated techniques. Plasma, platelet, and red cell DHLA and AA concentrations were estimated by gas-liquid chromatography after transmethylation to produce the methyl ester. The capacity of platelets in platelet-rich plasma to produce PGE_1 and PGE_2 was assessed by maximal stimulation with bovine thrombin (20 U/ml for 5 min at 37 C). After isolation[10] the prostaglandins were estimated by bioassay.

Experimental design—In the single-dose studies (table 1) the dose was taken at 9.30 am and blood samples were taken every one to two hours for six hours and then less often for up to 48 hours. In the sustained-dosing studies (table 1) blood was usually sampled four hours after the morning dose. Occasionally the effects of single doses were followed at the beginning and end of periods of sustained treatment. In all experiments the volunteers ate a breakfast low in saturated fats throughout the test period. No other dietary restriction was imposed. In the sustained-treatment studies in cases 1 and 2 vitamin E 3 mg was taken with each dose of DHLA and continued in a dose of 3 mg/day until blood sampling was stopped.

Drugs and regimens given during single-dose and sustained-dosing studies

Case No	Single-dose regimen	Sustained dosing studies	
		Drug	Dose
1	Placebo; 0·5g, 1g, 2g DHLA	DHLA	0·5g twice a day for 2 weeks 1g every morning for 1 week 1g twice a day for 1 week
2	Placebo; 1g, 2g DHLA	DHLA	1g twice a day for 10 days 0·5g twice a day for 10 days
3	Placebo; 0·1g, 1g DHLA	DHLA	0·1g every morning for 5 days
4	1g DHLEthyl ester; 0·1g DHLMethyl ester; 1g DHLA	DHLM-ethyl ester DHLA	0·1g every morning for 6 days 1g every morning for 8 days
5	2g DHLEthyl ester; 1g DHLMethyl ester		
6	0·1g DHLMethyl ester	DHLM-ethyl ester	0·1g every morning for 5 days
7	0·1g DHLMethyl ester	DHLM-ethyl ester	0·1g every morning for 6 days
8	1g DHLA		

Results

FATTY ACID AND PROSTAGLANDIN BIOCHEMISTRY

Single doses of DHLA were followed by dose-related increases in plasma DHLA : AA ratios (fig 2). Maximum ratios occurred three to five hours after dosing, with a secondary peak at six to 24 hours. Similar changes were seen in platelets. The two plasma peaks corresponded, respectively, to the appearance of DHLA in triglycerides and phosphatidyl choline. AA concentrations did not increase significantly in either plasma or platelets. DHLA esters seemed to be more poorly absorbed than the free acid. In the sustained-treatment studies DHLA penetrated additional lipid pools; cumulative increases in plasma and platelet DHLA : AA ratios were accompanied by increases in the ratio in red cell membranes. DHLA accumulated predominantly in phosphatidyl choline, but there was some accumulation in phosphatidyl ethanolamine. Small increases in the AA content of plasma, but not platelets, were sometimes detected.

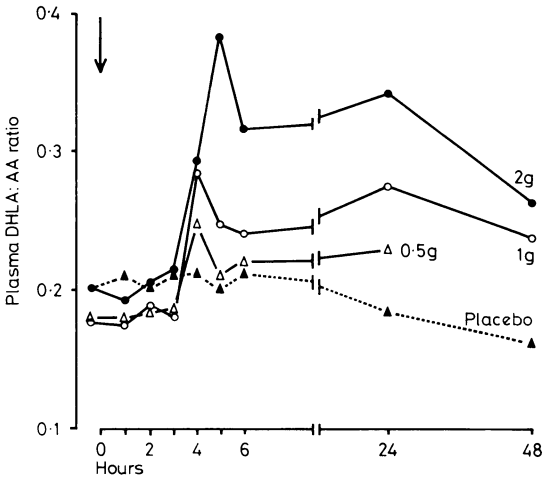

FIG 2—Case 1. Plasma DHLA : AA ratios in single-dose studies.

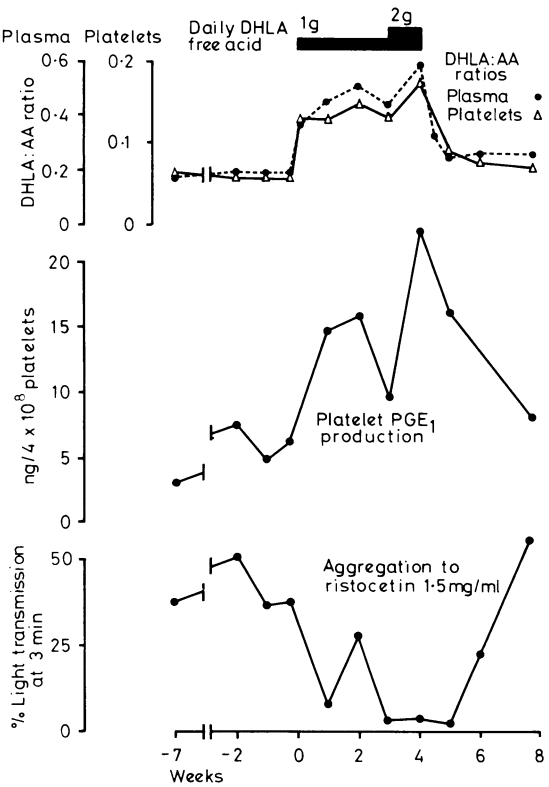

Fig 3—Case 1. Results of studies obtained during sustained-dosing study.

The capacity of platelets to produce PGE_1 and PGE_2 was estimated in eight of the single-dose studies. In each case there was a transient increase in PGE_1 production during the first six hours after dosing (55% mean rise); in six cases this increase was coincident with an increase in PGE_2 production (33% mean rise). These changes were not clearly dose related. Platelet prostaglandins were assayed during the three sustained dosing studies in which a daily dose of 1 g or more DHLA was used. Increases in PGE_1 production were more pronounced than after single doses; they were also sustained during the treatment period, coincident with changes in plasma and platelet DHLA : AA ratios (fig 3) and generally accompanied by parallel but less considerable increases in PGE_2 production.

HAEMOSTASIS

The principal changes in both the single-dose and the sustained-treatment studies were a decrease in plasma heparin-neutralising activity and an inhibition of platelet aggregation induced by adenosine diphosphate (ADP) (fig 4). Neither of these effects occurred during placebo studies. Single doses of DHLA or its esters were followed by a decrease in heparin-neutralising activity in 12 of the 15 studies.

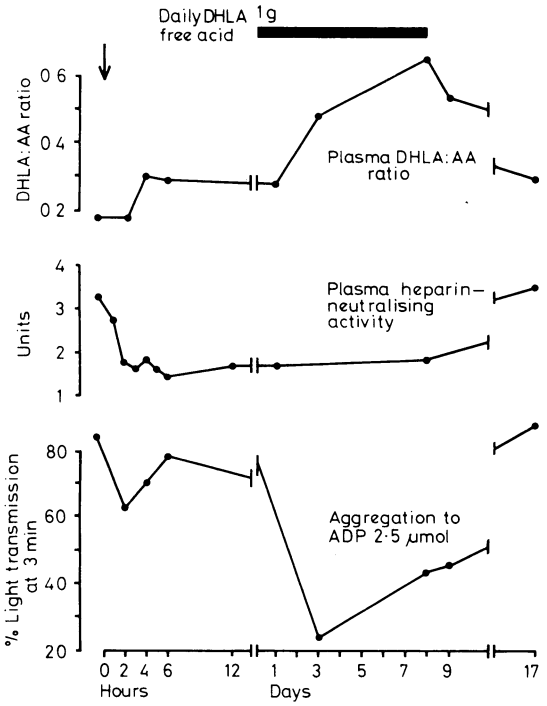

FIG 4—Case 4. Combined single-dose and sustained-dosing studies. 1 unit of heparin-neutralising activity is equivalent to 1 μg protamine sulphate.[9]

The two subjects who did not respond (cases 2 and 8) both had low basal levels of heparin-neutralising activity. The decrease in this activity was usually greatest four to six hours after dosing. There was no correlation between the size of the dose and the magnitude of the effect, which was clearly seen after all the single doses of 0·1 g. The duration of the effect, however, did seem to be related to the dose: after 0·1 g doses heparin-neutralising activity had always returned to baseline values by 24 hours, while the effects of larger doses were generally maintained at this time. Sustained treatment at all dose levels resulted in cumulative effects in six of the seven studies, the exception being the study on case 2. Positive effects were maintained for at least several days after dosing had been stopped.

ADP-induced platelet aggregation (0·25-2·5 μmol) was inhibited in nine of the 15 single-dose studies. The effect showed a similar time course to the reduction in heparin-neutralising activity but was less pronounced and usually did not persist for 24 hours. Effects were generally most pronounced after doses of 1 g or more. In the sustained treatment studies effects on aggregation varied between different subjects: when positive effects were seen these were greater than after single doses. Of the four 0·1-g studies aggregation was inhibited with free acid only in case 3, in whom a cumulative effect was apparent by the fifth day. In two of the three studies in which at least 1 g was given daily there was considerable and cumulative inhibition of ADP-induced aggregation (fig 4), which was coincident with increased platelet PGE_1 production.

In one of these two studies (case 1; fig 3) a wider panel of haemostatic tests was undertaken. His aggregation responses to ristocetin (1·5 mg/ml) and adrenaline (2·5 μmol) were also found to be inhibited. The response to ristocetin took almost four weeks to return to baseline values, and inhibition of ADP and adrenaline-induced aggregation was also still evident at this time. In this subject there were no effects on the bleeding time, in-vivo platelet adhesion,[11] platelet glass bead retention, factor VIII concentration, factor VIII-related antigen, or plasma concentrations of ristocetin cofactor. In the third study, where at least 1 g of DHLA was given daily (case 2) neither inhibition of aggregation nor a fall in heparin-neutralising activity could be detected despite significant increases in plasma and platelet DHLA : AA ratios. Although increases in platelet PGE_1 production and the PGE_1/PGE_2 ratio were observed in this subject, the increases were much smaller than those measured in the two other comparable studies.

CLINICAL MONITORING

There were no changes in chemical or haematological values that could be attributed to DHLA. Only once was a possibly related clinical adverse effect observed—a transient cough that occurred in case 5 about four hours after a single dose of 1 g DHLmethyl ester. This subject had a personal and family history of asthma and, interestingly, had had a similar reaction some years earlier after receiving intravenous PGE_1.

Discussion

This study shows that it is possible to manipulate platelet prostaglandin biosynthesis in man using the natural prostaglandin precursor for PGE_1 and that the changes induced are accompanied by potentially antithrombotic changes in haemostasis. DHLA was well tolerated when given orally, accumulated in lipid pools, and was accessible as a substrate for prostaglandin biosynthesis. Although increases in the capacity of platelets to produce PGE_1 may have accounted in part for the effects of DHLA on haemostasis, its precise mechanism of action has still to be defined. A puzzling finding was the rise in the capacity of platelets to produce PGE_2, which occurred in the absence of evidence of appreciable conversion of DHLA to AA, the biosynthetic precursor of PGE_2. One possible explanation is that DHLA or its metabolites, or both, may inhibit conversion of PGH_2 to the strongly proaggregatory TxA_2[1,12] and therefore redirect the conversion of PGH_2 to its prostanoate metabolites PGE_2, PGD_2, and PGI_2 (prostacyclin). The last two substances are potent inhibitors of platelet aggregation.[13,14].

The most impressive change in haemostasis was the reduction in heparin-neutralising activity produced by very small doses of DHLA. Plasma heparin-neutralising activity has been found to be high in various thrombotic states, including coronary heart disease and venous thrombosis.[8,15] The extent to which heparin-neutralising activity reflects basic pathological mechanisms is unknown; one view is that it represents, at least partly, the activity of platelet factor 4 released from platelets.[15] A reduction in heparin-neutralising activity may therefore indicate inhibition of the platelet release reaction. The role of platelet factor 4 in vivo is uncertain, but one possibility is that increased concentrations may have prothrombotic effects mediated through increased thrombin generation.

Although inhibition of platelet aggregation was less consistent than the fall in heparin-neutralising activity, the effect generally correlated well with changes in lipid and prostaglandin biochemistry, which at least partly explained the lack of response in case 2. Sim and McCraw[16] recently reported severe inhibition of ADP-induced platelet aggregation after 0·1-g doses of DHLmethyl ester and suggested that the methyl ester may be more active than the free acid. We do not agree with this suggestion and believe that the discrepancy between our findings

and those of Sim and McCraw is probably due to factors such as individual subject variation or differing experimental conditions. The most surprising finding in the aggregation studies was the inhibition of ristocetin-induced aggregation during the sustained dosing study in case 1. Lack of aggregation to ristocetin is characteristically associated with von Willebrand's disease, in which there is evidence of defective adhesion of platelets to subendothelium[17] and, in pigs, resistance to experimentally induced atherosclerosis.[18] Inhibition of ristocetin-induced aggregation therefore suggests that DHLA may have an inhibitory effect on platelet-vessel wall interaction, a probable early event in the formation of arterial thrombi. No other features of von Willebrand's disease were detected.

Diets low in saturated fats and rich in polyunsaturated fats (predominantly linoleic acid) both lower heparin-neutralising activity[19] and reduce platelet aggregability.[20] Since linoleic acid is a precursor of DHLA (fig 1) both these effects may be mediated through its conversion to DHLA. Such diets have also been reported to reduce mortality due to atherosclerosis,[21] and partial substitution of dietary saturated fats by polyunsaturated fats has been widely recommended as a means of reducing the risk of coronary heart disease.[22 23] Perhaps small doses of DHLA may be equally if not more effective than major dietary manipulations in preventing and treating these conditions.

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Reticulocytopenia and "absence" of red cell autoantibodies in immune haemolytic anaemia

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Summary

A raised reticulocyte count is common in patients with immune or autoimmune haemolytic anaemia, and the result of the direct antiglobulin test (DAGT) is usually positive because of IgG or IgG and complement components on the red cells. We report on three patients who had low reticulocyte counts when they were most anaemic, and in whom no red cell autoantibodies could be detected by the DAGT.

We postulate that reticulocytes may be selectively destroyed if antibodies are directed against antigenic sites on these young red cells, thus giving rise to a population of cells whose antigenic sites are poorly expressed. This theory might explain the low reticulocyte counts and the "absence" of antibodies (as tested by the DAGT) in such patients. Radioisotopic studies with ^{51}Cr and ^{59}Fe may provide useful information on the rate and sites of red cell destruction.

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Introduction

Increased destruction of red cells in "warm" immune or autoimmune haemolytic anaemia (AIHA) is commonly associated with a positive direct antiglobulin test (DAGT) result, usually because of the presence of either IgG alone or IgG and complement on the surface of the red cells. Some patients have all the clinical features of AIHA, but no antibody can be detected on the red cells by the direct antiglobulin test.[1-3] By using the sensitive antiglobulin consumption technique, however, small quantities of antibody belonging to the IgG class have been shown on the red cells of some patients with a negative DAGT result.[4] Most patients with AIHA have a raised reticulocyte count at presentation—a reflection of the high erythroid turnover that occurs to compensate for the anaemia and anoxaemia. Crosby and Rappaport,[5] however, noted the occurrence of reticulocytopenia in AIHA, and Pirofsky[3] also reported a low reticulocyte count in some patients with this disorder whose bone-marrow aspirate was normal or hyperplastic.

We report on three patients who had haemolytic anaemia with some unusual features. Neither IgG antibodies nor complement