

CLINICAL RESEARCH

Biosynthesis of thromboxane in patients with systemic sclerosis and Raynaud's phenomenon

I A G REILLY, L ROY, G A FITZGERALD

Abstract

Thromboxane A₂, the predominant cyclo-oxygenase product of arachidonic acid in platelets, is a potent vasoconstrictor and platelet agonist. Analysis of urinary metabolites by gas chromatography and mass spectrometry is a specific non-invasive method of measuring the biosynthesis of thromboxane that avoids the problem of platelet activation *ex vivo*. Excretion of the major urinary thromboxane metabolite, 2,3-dinor-thromboxane B₂, was significantly increased ($p < 0.001$) in 10 patients (nine women) with systemic sclerosis complicated by Raynaud's phenomenon compared with healthy controls (486 (SD 88) *v* 162 (38) ng/g creatinine) and increased further in the patients (to 1007 (212) ng/g creatinine) during application of a cold stimulus sufficient to induce digital vasoconstriction. Consistent with an increase in platelet-vascular interactions *in vivo*, excretion of a prostacyclin metabolite was also significantly increased ($p < 0.005$) in the patients with systemic sclerosis (248 (39) *v* 112 (10) ng/g creatinine) and tended to increase further on cooling.

Biosynthesis of thromboxane is increased in patients with systemic sclerosis and may exacerbate digital vasospasm that such patients develop when cold. This observation and the concomitant increase in the formation of prostacyclin provide a rationale for evaluating compounds that prevent the synthesis of thromboxane A₂ or inhibit its action while preserving the potential homeostatic role of prostacyclin.

Introduction

Raynaud's phenomenon is characterised by episodic, exaggerated constriction of the small digital arteries and arterioles in response to cold or emotional stress.¹ Studies of Raynaud's phenomenon

associated with systemic sclerosis have shown histological changes of intimal hyperplasia and severe fibrosis in the veins^{2,3} and an excessive reduction in peripheral blood flow after administration of vasoconstrictors.⁴ Thromboxane A₂, the predominant cyclo-oxygenase metabolite formed from arachidonic acid in the platelet,⁵ is a potent vasoconstrictor and platelet agonist. Because of this and the evolving therapeutic regimens designed selectively to inhibit synthesis of thromboxane⁶ or to antagonise its action,⁷ we studied the formation of thromboxane *in vivo* in patients with systemic sclerosis complicated by Raynaud's phenomenon. We used a specific, sensitive, and non-invasive approach to measure the biosynthesis of thromboxane to see whether it is increased in such patients and whether induction of Raynaud's phenomenon by exposure to cold is associated with a further increase.

Patients and methods

Ten patients (nine women and one man) with systemic sclerosis (scleroderma) complicated by symptomatic Raynaud's phenomenon participated in the study, which was approved by the Committee for the Protection of Human Subjects at Vanderbilt University Medical Center. Ages ranged from 36 to 60 (mean 45.4) years and each patient fulfilled the criteria of the American Rheumatism Association for a diagnosis of systemic sclerosis.⁸ Patients excluded had a clinical history or laboratory evidence (electrocardiogram and routine automated biochemical screen) of ischaemic heart disease or renal impairment (serum creatinine concentration $> 133 \mu\text{mol/l}$ ($> 1.5 \text{ mg/100 ml}$)) or were taking antiplatelet drugs. All patients studied were non-smokers and refrained from taking aspirin for at least two weeks before the study.

The patients and 10 healthy controls matched for age and sex were studied before and after central cooling had been induced with a cooling blanket (Aquatic-K-thermia, Gorman-Rupp, Bellville, Ohio). Cooling continued for 30 minutes or until Raynaud's phenomenon developed, which was observed in all patients. Care was taken to ensure that the maximum fall in the subject's normal oral temperature did not exceed 3°F. Blood samples were taken before cooling for measurement of plasma β thromboglobulin and platelet factor 4, serum thromboxane B₂, and platelet aggregation in response to arachidonic acid. Urine was collected for 24 hours before the study, followed by a second collection corresponding to the time of cooling, for measurement of the major urinary metabolites of thromboxane, 2,3-dinor-thromboxane B₂ and prostacyclin, 2,3-dinor-6-keto-prostaglandin F_{1 α} .

Biochemical analyses—2,3-Dinor-thromboxane B₂ and 2,3-dinor-6-keto-prostaglandin F_{1 α} were measured by stable isotope dilution assays using gas chromatography and mass spectrometry in the negative ion-chemical

Division of Clinical Pharmacology, Vanderbilt University, Nashville, Tennessee 37232, USA

I A G REILLY, MB, MRCP, research fellow

L ROY, MD, research fellow

G A FITZGERALD, MD, FRCPI, associate professor of medicine and pharmacology

Correspondence and requests for reprints to: Dr FitzGerald.

ionisation mode.^{9,10} Serum thromboxane B₂ generation in vitro was measured by radioimmunoassay using the method of Patrono *et al.*¹¹

Platelet function tests—Platelet aggregation was studied using a light transmission method in a Payton dual channel aggregometer.¹² The threshold aggregating concentration for arachidonic acid was determined (defined as the minimum concentration of arachidonic acid producing a greater than 70% increase in light transmission). The platelet α granule constituents were measured by radioimmunoassay (Amersham and Abbott Laboratories).

Statistical analyses were performed using a non-parametric method (the Wilcoxon rank sum test) to avoid making assumptions about the distribution of the variables.

Results

Basal excretion of 2,3-dinor-thromboxane B₂ was significantly increased in the patients with systemic sclerosis and Raynaud's phenomenon ($p < 0.001$) compared with the healthy controls (486 (SD 88) *v* 162 (38) ng/g creatinine). Basal excretion of 2,3-dinor-6-keto-prostaglandin F_{1 α} was also more than double in the patients compared with the controls (248 (39) *v* 112 (10) ng/g creatinine; $p < 0.005$). Both plasma β thromboglobulin concentration (57 (16) *v* 14 (2) μ g/l; $p < 0.005$) and platelet factor concentration (43 (14) *v* 6 (1) μ g/l; $p < 0.005$) were significantly higher in the patients. The threshold concentration for platelet aggregation in response to arachidonic acid was also significantly lower ($p < 0.005$) in the patients (0.16 (0.04) mmol/l) compared with the healthy subjects (0.38 (0.06) mmol/l). The ability of platelets to generate thromboxane A₂ as measured by serum thromboxane B₂ generation was normal in all of the patients (301 (72) *v* 276 (71) μ g/l) and confirmed that patients had avoided taking aspirin.

There was no significant effect of cooling on excretion of either of the metabolites in the controls (table). Typical clinical signs of Raynaud's phenomenon developed in the digits of all the patients with systemic sclerosis during cooling. There was a concomitant, significant increase in excretion of 2,3-dinor-thromboxane B₂ from 416 (80) to 1007 (212) ng/g creatinine ($p < 0.05$) during cooling (table) and a tendency for excretion of 2,3-dinor-6-keto-prostaglandin F_{1 α} to increase during cooling, though this did not attain significance.

Excretion of 2,3-dinor-thromboxane B₂, the major metabolite of thromboxane in urine,¹⁷ is a sensitive, specific, and non-invasive index of endogenous biosynthesis of thromboxane.¹⁸ Raised concentrations of this metabolite have been found in two additional models of platelet activation in vivo—namely, severe peripheral vascular disease¹⁹ and unstable angina.²⁰ In the present study further evidence suggesting platelet activation in vivo was provided both by the concomitant increase in plasma concentrations of platelet granule constituents and by the increase in endogenous formation of prostacyclin. Excretion of 2,3-dinor-6-keto-prostaglandin F_{1 α} is a non-invasive index of biosynthesis of prostacyclin that has been used to predict accurately the circulating concentrations of prostacyclin²¹ and reflect alterations in vascular biosynthesis of this eicosanoid.^{22,23} We previously showed considerable enhancement of biosynthesis of prostacyclin in patients with advanced peripheral vascular disease and platelet activation in vivo^{19,23} and a less pronounced increase in chronic cigarette smokers, who exhibited a correspondingly smaller increment in excretion of 2,3-dinor-thromboxane B₂.²⁴ Thus local formation of prostacyclin at sites of platelet-vascular interactions may have a homeostatic role in the setting of platelet activation in vivo.

We also found increased sensitivity of platelets to aggregation induced by arachidonic acid *ex vivo* in patients with systemic sclerosis. Additionally, plasma concentrations of the platelet granule constituents β thromboglobulin and platelet factor 4, were considerably raised in the patients. This may reflect the presence of platelet activation in vivo or increased sensitivity of platelets obtained from patients with systemic sclerosis to activation *ex vivo*. The clearance of platelet factor 4 from plasma is extremely rapid whereas the plasma half life of β thromboglobulin is more prolonged (about 100 minutes). Measurement of both platelet proteins in the same sample may, therefore, permit distinction between platelet activation in vivo and *ex vivo*.²⁵ The coincidental increase in platelet factor 4 suggests that the appreciable increase in β

Mean (SD) excretions of 2,3-dinor-thromboxane B₂ and 2,3-dinor-6-keto-prostaglandin F_{1 α} (ng/g creatinine) in patients with systemic sclerosis and healthy controls before and during cooling

	Patients with systemic sclerosis			Healthy controls		
	Before cooling	During cooling	Significance	Before cooling	During cooling	Significance
2,3-Dinor-thromboxane B ₂	416 (80)	1007 (212)	$p < 0.05$	162 (38)	284 (109)	NS
2,3-Dinor-6-keto-prostaglandin F _{1α}	248 (39)	362 (81)	NS	112 (10)	139 (18)	NS

Discussion

Endogenous biosynthesis of thromboxane A₂, a potent vasoconstrictor and platelet agonist, is significantly increased in patients with systemic sclerosis complicated by Raynaud's phenomenon. Moreover, a further increase in biosynthesis occurred when the syndrome was precipitated by exposure to cold. Such an observation is consistent with the presence of platelet activation in vivo and suggests that in patients with systemic sclerosis platelets are activated on application of a cold stimulus sufficient to induce Raynaud's phenomenon.

Previous studies of the biosynthesis of thromboxane in Raynaud's phenomenon have relied on measurement of plasma concentrations of thromboxane B₂.^{13,14} This variable is readily confounded by platelet activation in vitro. Whereas the ability of platelets to form thromboxane B₂ in serum is 300-400 μ g/l, concentrations of 100-200 ng/l are commonly reported in plasma.^{13,15} Thus activation of platelets to less than 0.1% of their capacity would be sufficient to account for these "endogenous" concentrations. That these concentrations indicate in vitro activation or are an analytical artefact, or both, is supported by non-invasive, maximal estimates of endogenous thromboxane B₂ concentrations in plasma in the range 1-2 ng/l derived from urinary metabolite excretion during infusion of thromboxane B₂.¹⁶ The problems of interpreting such indices of platelet activation in vivo led us to use an alternative approach in this

thromboglobulin noted in the patients may have derived, at least in part, from platelet release in vitro. This is not unexpected in view of the greater difficulty in obtaining blood samples free of stasis from such patients. As concentrations of platelet factor 4 were not measured in earlier studies, *ex vivo* artefact may perhaps have contributed to the raised concentrations of β thromboglobulin previously reported in patients with systemic sclerosis.^{26,27}

Clinical trials of drugs that inhibit thromboxane synthase have generally failed to show any appreciable benefit in Raynaud's phenomenon.^{13,14,28-30} These studies are, however, unlikely to have assessed the importance of thromboxane A₂ as a mediator in this disease. Firstly, studies both in vitro and in vivo have shown that prostaglandin endoperoxide substrate accumulates during inhibition of thromboxane synthase and substitutes for the action of thromboxane A₂,³¹⁻³³ thus limiting the clinical efficacy of thromboxane synthase inhibitors. Secondly, the regimens used in clinical trials of thromboxane synthase inhibitors incompletely inhibit biosynthesis of thromboxane.¹⁹ Maximal inhibition of platelet thromboxane generation is necessary to inhibit platelet activation dependent on thromboxane.^{34,35}

In conclusion, endogenous formation of thromboxane A₂ is enhanced in patients with systemic sclerosis, and precipitation of Raynaud's phenomenon by exposure to cold is associated with a further increase in biosynthesis of thromboxane. A coincidental increase in biosynthesis of prostacyclin in these patients may reflect

a homeostatic mechanism whereby the vasospastic and platelet aggregatory actions of thromboxane A₂ are attenuated. These results provide a rationale for evaluating treatment that inhibits thromboxane A₂ while preserving the synthesis of prostacyclin.

This study was supported by a grant (HL 30400) from the National Institutes of Health. LR was supported in part by grants from the Quebec Heart Foundation and the Tennessee Affiliate of the American Heart Association. GAF is an established investigator for the American Heart Association.

References

- Freedman RR, Ianni P. The role of cold and emotional stress in Raynaud's disease and scleroderma. *Br Med J* 1983;287:1499-502.
- Rodnan GP, Myerowitz RL, Justh GO. Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud's phenomenon. *Medicine (Baltimore)* 1980;59:393-407.
- Leroy EC. Pathogenesis of scleroderma (systemic sclerosis). *J Invest Dermatol* 1982;79:87-9.
- Halpern A, Kuhn PH, Shaftel HE, et al. Raynaud's disease, Raynaud's phenomenon and serotonin. *Angiology* 1960;11:151-67.
- Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci USA* 1975;72:2994-8.
- Needleman P, Raz R, Ferrendelli JA, Minkes M. Application of imidazole as a selective inhibitor of thromboxane synthetase in human platelets. *Proc Natl Acad Sci USA* 1977;74:1716-20.
- Jones RL, Wilson NH, Armstrong RA, Peesapati V, Smith GM. Effects of the thromboxane antagonist EP045 on platelet aggregation. *Adv Prostaglandin Thromboxane Leukotriene Res* 1983;11:345-50.
- American Rheumatism Association. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980;23:581-90.
- Lawson JA, Brash AR, Doran J, FitzGerald GA. Measurement of urinary 2,3-dinor-thromboxane B₂ and thromboxane B₂ using bonded phase phenyl boronic acid columns and capillary gas chromatography-negative ion chemical ionization-mass spectrometry. *Anal Biochem* 1985;150:463-70.
- FitzGerald GA, Lawson JA, Blair IA, Brash AR. Analysis of urinary metabolites of thromboxane and prostacyclin by negative ion-chemical ionization gas chromatography-mass spectrometry. *Advances in Prostaglandin Thromboxane and Leukotriene Research* 1985;15:87-90.
- Patrono C, Ciabattini G, Pinca E, et al. Low dose aspirin and inhibition of thromboxane B₂ production in healthy subjects. *Thromb Res* 1980;17:317-27.
- Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962;194:927-9.
- Luderer JR, Nicholas GG, Neumyer MM, et al. Dazoxiben, a thromboxane synthetase inhibitor, in Raynaud's phenomenon. *Clin Pharmacol Ther* 1984;36:105-15.
- Belch JFF, Cormie J, Newman P, et al. Dazoxiben, a thromboxane synthetase inhibitor, in the treatment of Raynaud's syndrome: a double blind trial. *Br J Clin Pharmacol* 1983;15:113-6.
- Hirsh PD, Hillis LD, Campbell WB, Firth BG, Willerson JT. Release of prostaglandins and thromboxane into the coronary circulation in patients with ischemic heart disease. *N Engl J Med* 1981;304:685-91.
- Patrono C, Ciabattini G, Pugliese F, Peirucci A, Blair IA, FitzGerald GA. Estimated rate of thromboxane secretion into the circulation of normal man. *J Clin Invest* 1986;77:590-4.
- Roberts LJ, Sweetman BJ, Oates JA. Metabolism of thromboxane B₂ in man: identification of twenty metabolites. *J Biol Chem* 1981;256:8384-93.
- FitzGerald GA, Pedersen AK, Patrono C. Analysis of prostacyclin and thromboxane A₂ biosynthesis in cardiovascular disease. *Circulation* 1983;67:1174-7.
- Reilly IAG, Doran J, Smith B, FitzGerald GA. Increased thromboxane biosynthesis in a human model of platelet activation: biochemical and functional consequences of selective inhibition of thromboxane synthase. *Circulation* (in press).
- FitzGerald DJ, Roy LR, FitzGerald GA. Enhanced prostacyclin and thromboxane A₂ synthesis in ischemic heart disease: non-invasive evidence of sporadic platelet activation in unstable angina. *Circulation* 1985;72:1688A.
- FitzGerald GA, Brash AR, Falardeau P, Oates JA. Estimated rate of prostacyclin secretion into the circulation of normal man. *J Clin Invest* 1981;68:1272-5.
- Roy L, Knapp H, Robertson RM, FitzGerald GA. Endogenous biosynthesis of prostacyclin during cardiac catheterization and angiography in man. *Circulation* 1985;71:434-9.
- FitzGerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. *N Engl J Med* 1984;310:1065-8.
- Nowak J, Murray JJ, Oates JA, FitzGerald GA. Platelet function and the in vivo formation of thromboxane A₂ and prostacyclin during chronic smoking and withdrawal in man [Abstract]. *Thromb Hemost* 1985;54:1029.
- Kaplan KL, Owen J. Plasma levels of β -thromboglobulin and platelet factor 4 as indices of platelet activation in vivo. *Blood* 1981;47:199-202.
- Zahavi J, Hamilton WAP, O'Reilly MJG, Leyton J, Cotton LT, Kakkar VV. Plasma exchange and platelet function in Raynaud's phenomenon. *Thromb Res* 1980;19:85-93.
- Kahaleh MB, Osborn I, Leroy EC. Elevated levels of circulating platelet aggregates and β -thromboglobulin in scleroderma. *Ann Intern Med* 1982;96:610-3.
- Ettinger WH, Wise RR, Schaffhauser D, Wigley FM. Controlled double-blind trial of dazoxiben and nifedipine in the treatment of Raynaud's phenomenon. *Am J Med* 1984;77:451-6.
- Coffman JD, Rasmussen HM. Effect of thromboxane synthetase inhibition in Raynaud's phenomenon. *Clin Pharmacol Ther* 1984;36:369-73.
- Jones EW, Hawkey CJ. A thromboxane synthetase inhibitor in Raynaud's phenomenon. *Prostaglandins Leukotrienes Med* 1983;12:67-71.
- Bertele V, de Gaetano G. Potentiation by dazoxiben, a thromboxane synthetase inhibitor, of platelet aggregation inhibitory activity of a thromboxane receptor antagonist and of prostacyclin. *Eur J Pharmacol* 1982;85:331-3.
- FitzGerald GA, Reilly IAG, Pedersen AK. The biochemical pharmacology of thromboxane synthase inhibition in man. *Circulation* 1985;72:1194-201.
- FitzGerald DJ, Frassetta JE, Jackson EK, FitzGerald GA. Prostaglandin endoperoxides limit the platelet inhibitory effects of thromboxane synthase inhibitors in vivo. *Clin Res* 1985;33:282.
- Di Minno G, Silver MJ, Murphy S. Monitoring the entry of new platelets into the circulation after ingestion of aspirin. *Blood* 1983;61:1081-5.
- FitzGerald GA, Oates JA, Hawiger J, et al. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *J Clin Invest* 1983;71:676-88.

(Accepted 6 February 1986)

Screening for intrauterine growth retardation using ratio of mid-arm circumference to occipitofrontal circumference

N J MEADOWS, J TILL, A LEAF, E HUGHES, B JANI, V LARCHER

Abstract

Uncritical application of standard weight percentile charts, derived from white infants, to infants from different ethnic groups may result in an overestimate of the incidence of intrauterine growth retardation in those groups. The ratio of mid-arm circumference to occipitofrontal circumference was studied in 194 babies (49 Asian, 58 black, and 87 white). In contrast with birth weight the ratio did not vary among the ethnic groups; it was also independent of sex. In a prospective study of

64 neonates whose weight was below the 10th percentile on standard charts the ratio of mid-arm circumference to occipitofrontal circumference was a more accurate predictor than weight of those infants who would develop symptoms associated with intrauterine growth retardation.

The ratio of mid-arm circumference to occipitofrontal circumference therefore provides a simple, accurate, and cheap way of assessing intrauterine growth retardation in areas with a large multiethnic population, where birth weight varies greatly.

Department of Child Health, Salvation Army, Mothers' Hospital, London E5
 N J MEADOWS, MB, MRCP, lecturer
 J TILL, MB, BSC, senior house officer
 A LEAF, MB, MRCP, senior house officer
 E HUGHES, MB, MRCP, senior house officer
 B JANI, MB, DCH, senior house officer
 V LARCHER, MB, MRCP, consultant paediatrician

Correspondence to: Dr N J Meadows, Queen Elizabeth Hospital for Children, London E2 8PS.

Introduction

The mean birth weight of white infants differs from that of infants born to mothers from other ethnic groups, especially Asian.¹⁻³ Because established percentile charts relate to white infants, the criterion of weight alone cannot be used to identify accurately intrauterine growth retardation in infants from other ethnic groups. We measured the ratio of mid-arm circumference to occipitofrontal circumference in normal full term babies from different ethnic