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Iron chelators inhibit human platelet aggregation, thromboxane A_2 synthesis and lipoxygenase activity

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The iron chelators desferrioxamine and 1,2-dimethyl-3-hydroxypyrid-4-one (L1) inhibited human platelet aggregation in vitro as well as thromboxane A_2 synthesis and conversion of arachidonate to lipoxygenase-derived products. Non-chelating compounds related to L1 were without effect on cyclooxygenase or lipoxygenase activity. Since both cyclooxygenase and lipoxygenase are iron-containing enzymes, it is suggested that the inhibition of platelet function by these iron chelators may be related to the removal or binding of iron associated with these enzymes. These iron chelators may therefore be of potential therapeutic value as platelet antiaggregatory agents and of possible use in the treatment of atherosclerotic and inflammatory joint diseases.

Iron; Dimethyl-3-hydroxypyrid-4-one, 1,2-; Desferrioxamine; Lipoxygenase; Platelet aggregation; Thromboxane A_2

1. INTRODUCTION

Despite the fact that the main clinical use of the iron chelators desferrioxamine (DFO) and 1,2-dimethyl-3-hydroxypyrid-4-one (L1) is the treatment of iron overload [1], these chelators may have other potential clinical uses where iron-containing enzymes [2] regulate metabolites which contribute to the pathogenesis of disease. For example, a recent study has demonstrated that DFO and L1 inhibit prostanoid synthesis by vascular tissue through binding of iron associated with cyclooxygenase. Since certain prostanoids are pro-inflammatory, it was suggested that iron chelators may prove beneficial in the treatment of inflammatory and other diseases through inhibition of cyclooxygenase [3]. It has also been proposed that platelets and their released products may play a role in the pathophysiology of inflammatory and atherosclerotic disease [4–7].

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With regard to atherosclerosis, it has been suggested that platelets may adhere to existing atherosclerotic plaques, releasing substances (thromboxane A_2 (TXA_2), lipid peroxides, growth factors) whose chemotactic, vasoconstrictor and mitogenic properties may further accelerate the atherogenic process [5–10]. Cyclooxygenase and lipoxygenase also possess the potential to generate other oxygen free radicals, which may also contribute to platelet-mediated vascular pathogenesis [11,12].

Although little is known of the role of platelets in inflammatory joint disease, it is well established that prostanoids and leukotrienes play a cardinal role in the aetiology of this disorder [3,7]. In this context, it has been demonstrated that rheumatoid synovial membranes contain large amounts of iron [13], and that intraplatelet iron is elevated in patients with ankylosing spondylitis [14]. It is thus possible that there is an aetiological link between elevated intraplatelet iron, increased cyclooxygenase and lipoxygenase activity and proinflammatory hydroperoxyeicosanoid synthesis.

We therefore investigated the effect of DFO and

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L1 and of non-chelating structural analogues of L1 on human platelet aggregation and TXA_2 synthesis. Platelet lipoxygenase activity in the presence of these chelators was also assessed since products of this iron-containing enzyme are also thought to play a role in the pathogenesis of inflammation and atherosclerosis [7].

2. MATERIALS AND METHODS

2.1. *Iron chelators*

1,2-Dimethyl-3-hydroxypyrid-4-one (L1) was prepared as previously described [15]. 3-Hydroxy-2-methylpyr-4-one (maltol), 4-hydroxy-6-methylpyr-2-one and 2,4-dihydropyridine were obtained from Aldrich Chemical Company, Gillingham, Dorset, England. Desferrioxamine was a gift from Ciba Geigy England, Horsham, Sussex, England.

2.2. *Platelet aggregation and thromboxane A_2 release*

Blood was collected (from an antecubital vein) with minimal stasis from 10 healthy volunteers who denied taking any drugs, particularly nonsteroidal antiinflammatory drugs, for the previous 2 weeks. The blood was anticoagulated with 3.8% (w/v) trisodium citrate (9 parts blood to 1 part citrate) as previously described [16]. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by centrifugation at room temperature. Aggregations were carried out in Chronolog dual channel aggregometers (Coulter Electronics, Luton, Beds., England). Aggregation was expressed as the percentage fall in absorbance between PRP and PPP 3 min after adding aggregating agents (collagen, adenosine diphosphate (ADP) and adrenaline). The agonist concentration selected was that which generated just submaximal aggregation (ADP: median agonist concentration 6 μmol/l; range 2–10 μmol/l; adrenaline: 3 μmol/l; range 0.5–5 μmol/l; collagen: 0.7 mg/l; range 0.3–1.0 mg/l). Iron chelators dissolved in autologous plasma were added to the PRP 5 min prior to the addition of agonists. As a further control, the effect of iron chelators on aspirin-treated platelets was also investigated. Measurement of TXA_2 release was carried out as previously described [16]. Briefly, 3 min after adding agonists the PRP was added to absolute ethanol to stop the reaction. This sample was stored at $-70°C$ until measurement of thromboxane B_2 (TXB_2; the spontaneous, stable breakdown product of thromboxane A_2) in a single batch using a specific radioimmunoassay [16].

2.3. *Platelet thromboxane A_2 synthesising capacity*

PRP aliquots were centrifuged in an Eppendorf ultracentrifuge and platelet pellets were washed with normal saline. The pellets were then frozen to $-70°C$ and thawed, and Krebs Ringer bicarbonate buffer, including varying concentrations of the iron chelators, was added. The samples were then sonicated for 10 s in a Soniprep 150 sonicator (MSE, Crawley, Sussex, England). The pellets were then incubated at 37°C for 1 h after which time an aliquot of the supernatant was taken for estimation of TXB_2 by radioimmunoassay as previously described [17]. This procedure is an index of total synthesising capacity of TXA_2 by platelets [17].

2.4. *Platelet lipoxygenase activity*

Tris buffer (100 mmol·l^{-1}, pH 7.4), containing 1 mM EDTA (1 mmol·l^{-1}), varying concentrations of iron chelators and 50 nCi [^{14}C]arachidonate (1 Ci·$mmol^{-1}$; New England Nuclear, Dreieich, FRG) was added to the pellets as described above. The pellets were then sonicated and incubated at 37°C for 30 min. Incubates were extracted with ethyl acetate, and the lipoxygenase and cyclooxygenase products and the unchanged arachidonate (AA) were separated by thin-layer chromatography [18]. Zones corresponding to hydroxy- and hydroperoxyeicosatetraenoic acids (HETE and HPETE) were removed and counted for radioactivity, and percentage conversion of labelled arachidonate into lipoxygenase products was calculated.

3. RESULTS AND DISCUSSION

Both DFO and L1 inhibited platelet aggregation (table 1) and concomitant TXA_2 release (table 2) in dose-dependent manners. The relative sensitivity of the aggregatory agonists (adrenaline > collagen > ADP) to the iron chelators indicates that the inhibition of platelet aggregation is likely to be due to an inhibition of TXA_2 synthesis [16,17,19]. For example, the same pattern of in-

Table 1

Effect of 1,2-dimethyl-3-hydroxypyrid-4-one (L1) and desferrioxamine (DFO) on agonist-stimulated platelet aggregation

	Control	1	2	4	8
L1 (mmol·l^{-1})					
ADP	65 (40–70)	68 (45–75)	60 (37–65)	24* (0–42)	10* (0–20)
Adrenaline	77 (42–90)	74 (70–80)	35* (22–57)	8* (2–12)	0* (0)
Collagen	68 (40–77)	68 (56–90)	64 (7–89)	0* (0–62)	0* (0)
DFO (mmol·l^{-1})					
ADP	64 (40–77)	64 (27–54)	35* (0–54)	32* (15–39)	
Adrenaline	62 (50–88)	47* (30–80)	31* (0–71)	10* (0–19)	
Collagen	65 (40–93)	62 (25–89)	20* (0–82)	0* (0–62)	

Platelet aggregation (% fall in absorbance) is expressed as median and (range). PRP was prepared from 10 healthy control subjects. For agonist concentrations, see text. * $P < 0.01$; control vs drug. Wilcoxon rank sum test for paired samples

Table 2

Effect of 1,2-dimethyl-3-hydroxypyrid-4-one (L1) and desferrioxamine (DFO) on agonist-stimulated TXA_2 release by human platelets

	Control	1	2	4	8
L1 ($mmol \cdot l^{-1}$)					
ADP	14 (9–16)	12 (8–14)	7* (5–12)	3* (3–5)	0* (<1)
Adrenaline	20 (12–23)	18 (12–20)	8* (1–12)	1* (0–2)	0* (<1)
Collagen	27 (22–31)	26 (20–30)	20* (15–22)	10* (8–13)	7* (4–9)
DFO ($mmol \cdot l^{-1}$)					
ADP	10 (8–13)	5* (3–8)	0* (<1)	0* (<1)	0* (<1)
Adrenaline	18 (10–20)	11* (5–14)	4* (2–6)	0* (<1)	0* (<1)
Collagen	24 (17–28)	11* (9–15)	4* (1–3)	0* (<1)	0* (<1)

TXA_2 release ($ng \cdot ml^{-1}$ PRP) is expressed as median and (range). PRP was prepared from 10 healthy control subjects. For agonist concentrations, see text. * $P < 0.01$; control vs drug. Wilcoxon rank sum test for paired samples

Table 3

Effect of 1,2-dimethyl-3-hydroxypyrid-4-one (L1) and desferrioxamine (DFO) on agonist-stimulated and aspirin (ASA) inhibited platelet aggregation

	Control	DFO (2 mmol/l)	ASA (0.55 mmol/l)	DFO + ASA (2 mmol/l + 0.55 mmol/l)
ADP	63 (60–69)	33 (31–54)	45 (34–53)	32 (31–44)
Adrenaline	75 (60–80)	5 (5–8)	7 (3–9)	5 (2–10)
Collagen	64 (58–70)	20 (10–22)	0 (0)	0 (0)
	Control	L1 (4 mmol/l)	ASA (0.55 mmol/l)	L1 + ASA (4 mmol/l + 0.55 mM)
ADP	73 (65–80)	51 (46–56)	60 (57–62)	48 (40–55)
Adrenaline	72 (70–74)	10 (9–11)	15 (12–18)	10 (8–11)
Collagen	84 (67–90)	20 (10–67)	0 (0–18)	0 (0–22)

Platelet aggregation (% fall in absorbance) is expressed as median and (range). PRP was prepared from four healthy control subjects. For agonist concentrations, see text

hibition of platelet aggregation has been observed with aspirin [19], indomethacin [20], tiaprofenic acid [20], ethanol [16] and the 'classical' TXA_2 synthase inhibitor, 1-*N*-butyl imidazole [19]. Furthermore, in the present study, inhibitory doses of DFO and L1 did not markedly alter the characteristic inhibitory action of aspirin on ADP-, adrenaline- or collagen-stimulated aggregation (table 3), consolidating the conclusion that these chelators are inhibiting aggregation through inhibition of TXA_2 synthesis. This interpretation was confirmed in the platelet pellets, since both DFO and L1 inhibited the maximal TXA_2-synthesising capacity of freeze-thawed-sonicated platelets (fig.1). This latter method of assessing platelet TXA_2 synthesis obviates a priori aggregation and is an index of the activity of TXA_2 synthesising enzyme, viz. phospholipase A_2, cyclooxygenase and TXA_2 synthase [17].

Since there is no evidence that either PLA_2 or TXA_2 synthase require trivalent cations and cyclooxygenase contains haem [21,22], it is sug-

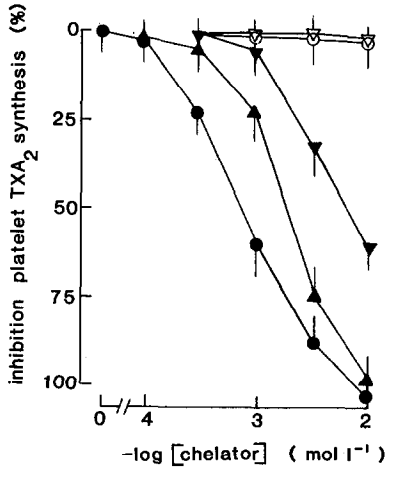

Fig.1. Effect of DFO (●), L1 (▲), maltol (▼), 4-hydroxy-6-methylpyr-2-one (○) and 2,4-dihydroxypyridine (▽) on human platelet TXA_2 synthesis of human freeze-thawed-sonicated platelets. Each point represents mean ± SE, $n = 10$. IC_{50}s: L1, 2×10^{-3} $mol \cdot l^{-1}$; DFO, 7×10^{-4} $mol \cdot l^{-1}$; maltol, 8×10^{-3} $mol \cdot l^{-1}$.

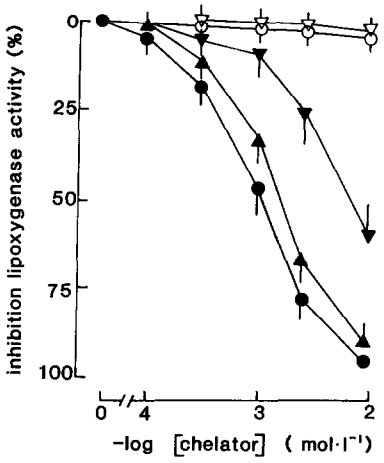

Fig.2. Effect of DFO (●), L1 (▲), maltol (▼), 4-hydroxy-6-methylpyr-2-one (○) and 2,4-dihydroxypyridine (▽) on conversion of arachidonate to HETE and HPETE by human platelets. Each point represents mean ± SE, n = 10. IC_{50}s: L1, 1.6×10^{-3} mol·l^{-1}; DFO, 1.2×10^{-3} mol·l^{-1}; maltol, 8×10^{-3} mol·l^{-1}.

gested that the iron chelators are inhibiting platelet aggregation through removal (or binding) of iron associated with cyclooxygenase in a manner similar to that previously observed with vascular prostacyclin synthesis [3]. That DFO and L1 are acting non-specifically on cyclooxygenase and lipoxygenase is discounted since non-chelating compounds related to L1 were without effect (figs 1 and 2) and maltol, a weaker chelator than DFO or L1 was of intermediate potency (figs 1 and 2). Furthermore, the presence of trivalent cations, iron and aluminium, reversed the inhibitory action of DFO and L1 on platelet TXA_2 synthesis (table 4), presumably through negation of the iron binding capacity of the chelators. Although not proving the involvement of iron, these data consolidate the above point that L1 and DFO are not acting non-specifically on cyclooxygenase.

The present study also demonstrates that DFO and L1 inhibit platelet lipoxygenase activity (fig.2), an action which is also reversed by the addition of exogenous iron and aluminium (table 4). Since it has been established that human platelet lipoxygenase contains non-haem iron [23], it is likely that these chelators are inhibiting lipoxygenase activity through the removal or binding of iron associated with this enzyme.

In contrast to the present findings, it has been reported by Morgan et al. [24] that DFO enhances the conversion of exogenous AA to PGI_2 but diminishes lipoxygenase activity in homogenates of the rat uterus [24]. In a previous study, however, we found that AA-stimulated PGI_2 by intact rat aortic tissue was inhibited by DFO and L1 [3]. Thus, the only apparent difference in methodology which might explain this disparity is that Morgan et al. investigated homogenised tissue.

In addition to the inhibition of free radical damage induced by non-protein-bound iron [25], the inhibition of platelet activity by these chelators may also be useful in the treatment of thrombotic, atherosclerotic and inflammatory diseases via several mechanisms: firstly, as antithrombotic agents through inhibition of platelet aggregation and TXA_2 release; secondly, in atherogenesis as inhibitors of platelet adhesion, growth factor release and lipid peroxidation; thirdly, as inhibitors of proinflammatory hydroperoxyeicosanoid syn-

Table 4

Effect of Fe^{3+} and Al^{3+} on DFO- and L1-inhibited platelet TXA_2 and lipoxygenase activity (percentage inhibition ± SD, n = 10)

	3 mM L1	3 mM L1 + 1 mM Fe^{3+}	3 mM L1 + 3 mM Fe^{3+}	3 mM L1 + 1 mM Al^{3+}	3 mM L1 + 3 mM Al^{3+}
TXA_2 inhibition	75 ± 10	50 ± 12	10 ± 4	48 ± 6	8 ± 1
Lipoxygenase inhibition	80 ± 6	45 ± 8	12 ± 2	53 ± 8	8 ± 2
	2 mM DFO	2 mM DFO + 1 mM Fe^{3+}	2 mM DFO + 2 mM Fe^{3+}	2 mM DFO + 1 mM Al^{3+}	2 mM DFO + 2 mM Al^{3+}
TXA_2 inhibition	85 ± 10	40 ± 4	2 ± 0.2	39 ± 3	2 ± 0.1
Lipoxygenase inhibition	81 ± 12	38 ± 3	4 ± 0.1	42 ± 4	8 ± 0.4

thesis; and finally, through inhibition of toxic free radical generation. These mechanisms may apply not only to platelets but also to leukocytes, since these cells also release hydroperoxyeicosanoids [6].

It should be noted, however, that DFO, L1 and possibly other chelators can only be used in the above conditions if sufficient therapeutic non-toxic concentration ranges are reached in vivo. The indications are that in some cases this may have occurred with DFO, where doses of up to 235 mg/kg were used in thalassaemia patients [26], since it is known that plasma concentrations exceeding 300 μmol/l could be achieved with a dose of DFO of 80 mg/kg in haemodialysis patients [27]. The design and screening of more chelators of the α-ketohydroxypyridine [28–30] or the desferrioxamine [31] classes, which could be more specific than L1 and DFO in inhibiting lipoxygenase and cyclooxygenase at lower doses and their interaction with other inhibitors of these enzymes, is currently under investigation.

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