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EFFECTS OF DESFERRIOXAMINE ON EICOSANOID PRODUCTION IN TWO INTACT CELL SYSTEMS

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Abstract—Desferrioxamine is an iron-chelating agent used in the treatment of iron overload. It is a powerful inhibitor of iron-dependent radical reactions and has been used to test for the involvement of such reactions in animal models of human disease. Recent proposals that desferrioxamine inhibits lipoxygenase and stimulates cyclo-oxygenase were examined by testing its action upon eicosanoid production in rat caecal fragments and elicited rat peritoneal leukocytes. Under certain experimental conditions, higher concentrations of desferrioxamine (up to 0.5 mM) enhanced eicosanoid production in leukocytes. Desferrioxamine concentrations ≥500 μM also stimulated cyclo-oxygenase activity in caecal fragments. Although these results show that high concentrations of desferrioxamine can augment eicosanoid production under certain circumstances, the effects are likely not to be signfiicant therapeutically in view of the concentrations of desferrioxamine achieved during *in vivo* use in humans.

Desferal® (desferrioxamine B methanesulphonate) is an effective iron-chelating agent used to treat and prevent iron-overload conditions such as may occur after transfusion in thalassaemia [1–3]. Doses of 50–60 mg/kg body weight administered by subcutaneous or intravenous routes appear fairly safe in the treatment of iron-overload [4], but higher doses have been associated with ocular abnormalities [4, 5] and with auditory changes [6, 7]. Toxic effects of desferrioxamine appear at lower doses when it is administered to patients with sub-normal blood iron levels, such as those suffering from rheumatoid arthritis [8, 9].

Desferrioxamine is also a powerful inhibitor of iron-dependent free radical reactions, such as lipid peroxidation and hydroxyl radical formation [10, 11] and also has radical-scavenging properties [12]. It has thus been widely used to test for the importance of iron-dependent radical reactions in animal models of several human diseases (reviewed in Ref. 13), including models of inflammatory joint disease [14, 15]. However, attribution of the anti-inflammatory effects of desferrioxamine to interference with iron-dependent free radical reactions has been questioned because of recent proposals that the drug inhibits lipoxygenase [15] and stimulates cyclo-oxygenase [16]. However, no experimental data were presented in the first report [15], and high concentrations of desferrioxamine (≥500 μM) were needed to obtain an effect in the second [16].

In the present paper, we have investigated in detail the effect of desferrioxamine on arachidonic acid metabolism. Rat caecal fragments [17] and elicited rat peritoneal leukocytes [18] were used as test systems, rather than homogenates [16] in which cellular integrity has been completely lost. Our preparations have the added advantages that eicosanoid formation can be studied without the need for addition of exogenous arachidonic acid, and that products of both cyclo-oxygenase and lipoxygenase pathways are generated.

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MATERIALS AND METHODS

Eicosanoid generation by elicited rat peritoneal leukocytes [18] and rat caecal fragments [17, 19] was assayed using methods described in detail elsewhere. In brief, mixed rat peritoneal leukocytes (*ca.* 70% polymorphonuclear neutrophils, the remainder mononuclear cells, >95% viability by Trypan blue exclusion) were obtained from female Wistar rats 20 hr after an i.p. injection of 6% (w/v) glycogen in sterile 0.9% (w/v) saline. After removing any contaminating erythrocytes by lysis, the cells were isolated by differential centrifugation and resuspended in modified Hanks' balanced salt solution (HBSS) containing 1.26 mM Ca^{2+}, 0.9 mM Mg^{2+} and 10 mM L-cysteine. Aliquots of 0.5 ml cells at 5×10^6 cells/ml were preincubated for 10 min with test drug and stimulated by adding 10^{-5} M A23187 or 10^{-5} M *N*-formyl-Met-Leu-Phe (FMLP) for a further 10 min at 37°. The cells were pelleted and the supernatants subjected to radioimmunoassay for leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and thromboxane B_2 (TXB_2). TXB_2 and 6-keto $PGF_{1\alpha}$ are the stable hydrolysis products of thromboxane A_2 and prostacyclin, respectively. All assays involved polyclonal antibodies of appropriate specificity raised in rabbits with cross-reactivities as follows: anti-LTB_4, LTB_4 100%, 20-OH and 20-COOH metabolites of LTB_4 0.3% and 0.1%, other hydroxyarachidonate metabolites, prostaglandins and LTB_4 isomers less than 0.5% excepting all-*trans* LTB_4 (2.2%); cross-reactivities against PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 for the anti-PGE_2 were 100%, 0.7% and 2.4%, for the anti-6-keto-$PGF_{1\alpha}$ were 4.0%, 100% and 1.4%, and

for the anti-TXB_2 were 0.2%, 0.1% and 100%, respectively.

In some additional experiments, eicosanoid generation by the peritoneal cells was measured by adding [^{14}C]-arachidonic acid as an exogenous substrate: to suspensions of 10^7 cells in 0.5 ml, 0.125 μCi arachidonate (4.3 μM) and 1 μM A23187 were added. After 10 min the products were extracted at pH 3.5 with ethyl acetate. The dried residues were dissolved in methanol and applied to foil-backed silica gel TLC sheets and the amounts of arachidonic acid (R_f 0.68), 5-hydroxyeicosatetraenoic acid (5-HETE, R_f 0.57), LTB_4 (R_f 0.46), TXB_2 (R_f 0.28), PGE_2 (R_f 0.23) and 6-keto $PGF_{1\alpha}$ (R_f 0.12) quantified by sectioning the sheets after developing the chromatogram in a solvent containing ethyl acetate/formic acid 80:1 (v/v). The entire chromatogram was cut up and counted; thus avoiding the possibility of neglecting other important metabolites corresponding to eicosanoids migrating at points different from those of the authentic standards listed above.

In separate control experiments using both radio-TLC and RIA it was established that the rat peritoneal leukocytes do not convert LTB_4 to more polar 20-OH and 20-COOH compounds after either 5 min or more prolonged incubation (30–60 min), whereas in human peripheral neutrophil leukocytes such conversion was found to occur. Further, it was found that there was no material present on the radiochromatograms corresponding to the positions of those isomers of LTB_4 formed by non-enzymatic hydrolysis of LTA_4.

Caeca from male Wistar or Sprague–Dawley rats were cleaned and used fresh or after freezing for 2–8 wk in saline; the tissue was cut into approximately equal pieces (15–45 mg wet weight), preincubated at 4° for 60 min in buffer or drug-containing solutions, and then transferred to a similar solution for 15 min incubation at 37° in a shaking water bath operating at 60 strokes/min. After removing and weighing the caecal fragments, the supernatants were subjected to radioimmunoassay as above.

Radioisotopes were from Dupont (NEN) (Stevenage, Herts, U.K.) and Amersham International (Little Chalfont, Bucks, U.K.), unlabelled eicosanoids and other reagents were from the Sigma Chemical Company (Poole, Dorset, U.K.) unless otherwise indicated. Desferrioxamine B methanesulphonate and mefenamic acid were kind gifts from CIBA-Geigy (Horsham, Sussex) and Parke, Davis & Company (Pontypool), respectively. Iron-loaded desferrioxamine (ferrioxamine) was prepared by mixing freshly prepared solutions of desferrioxamine and ferric chloride in the molar ratio 1:0.9 immediately before use, thereby avoiding any risk of the presence of excess iron.

RESULTS

Desferrioxamine enhanced the generation of prostacyclin by rat caecal fragments (Table 1) in a dose-dependent manner, with small but significant effects at 50 μM and 500 μM and increasing up to the largest dose tested, 5 mM (Fig. 1). Frozen/thawed fragments released more prostacyclin than freshly used tissue (Table 1), but desferrioxamine at 5 mM caused increased release in both kinds of preparation, by 1.6–2.9-fold in frozen tissue and 1.8–4.6-fold in fresh tissue. Adrenaline has previously been shown to enhance prostacyclin generation in this system [19]; at 5 mM it was a more effective agent than desferrioxamine (Table 1). The non-steroidal cyclo-oxygenase inhibitor, mefenamic acid, substantially inhibited prostacyclin release at 5 μM concentration (Table 1). The production of other eicosanoids released from rat caecal fragments in amounts smaller than that of prostacyclin was also enhanced by desferrioxamine. For example, 5 mM desferrioxamine and 5 mM adrenaline increased the output of PGE_2 from 0.07 ± 0.01 ng/mg/15 min to 0.40 ± 0.10 ng/mg/15 min and 0.36 ± 0.08 ng/mg/15 min, respectively, and the output of TXB_2 from 0.29 ± 0.05 ng/mg/15 min to 0.46 ± 0.06 ng/mg/15 min and 2.16 ± 0.35 ng/mg/15 min, respectively. The generation of leukotriene B_4 (LTB_4) was not

Table 1. Effect of desferrioxamine, its ferric chelate and other drugs on prostacyclin production by rat caecal fragments

Condition	Control	6-keto $PGF_{1\alpha}$ generation, ng/mg/15 min			
		DFO 5 mM	DFO/Fe^{3+} 5 mM	Adrenaline 5 mM	Mefenamic acid 5 μM
Frozen	0.75 ± 0.08	1.81 ± 0.32*	nt	nt	nt
Frozen	1.11 ± 0.20	2.90 ± 0.36*	nt	5.78 ± 0.68*	0.26 ± 0.05*
Frozen	1.09 ± 0.15	1.77 ± 0.26*	0.58 ± 0.13*	2.45 ± 0.67*	0.46 ± 0.11*
Frozen	0.37 ± 0.05	1.07 ± 0.26*	nt	2.18 ± 0.28*	0.19 ± 0.05*
Frozen	1.58 ± 0.21	3.22 ± 0.60*	1.01 ± 0.21	3.18 ± 1.04	1.09 ± 0.16
Frozen	0.97 ± 0.15	2.14 ± 0.42*	0.39 ± 0.15*	2.03 ± 0.25*	0.44 ± 0.13*
Fresh	0.10 ± 0.03	0.18 ± 0.06	nt	0.36 ± 0.10*	0.04 ± 0.01
Fresh	0.25 ± 0.06	1.16 ± 0.35*	0.35 ± 0.11	1.23 ± 0.54*	0.22 ± 0.08
Fresh	0.28 ± 0.06	0.51 ± 0.14	0.25 ± 0.06	nt	nt

Results show mean ± SEM for duplicate determinations of amounts released from 10 fragments incubated independently (control) or 5 fragments (drug-treated). * indicates significant difference from control, $P < 0.05$, Student's unpaired t-test. DFO = desferrioxamine; DFO/Fe^{3+} = iron-loaded desferrioxamine (ferrioxamine); nt = not tested.

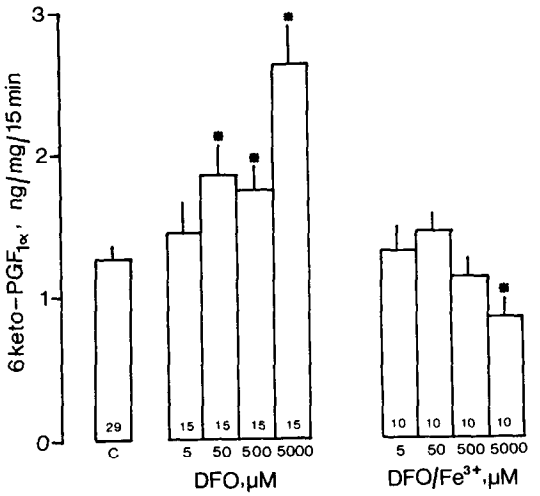

Fig. 1 Effect of desferrioxamine and iron-loaded desferrioxamine (ferrioxamine) on prostacyclin generation by rat caecal fragments. These results were obtained using frozen/thawed caecal fragments and show mean ± SEM; the number of fragments used at each concentration is shown at the base of the bars. *Indicates significant difference ($P < 0.05$) from control, Student's t-test. C = control, DFO = desferrioxamine, DFO/Fe^{3+} = ferrioxamine.

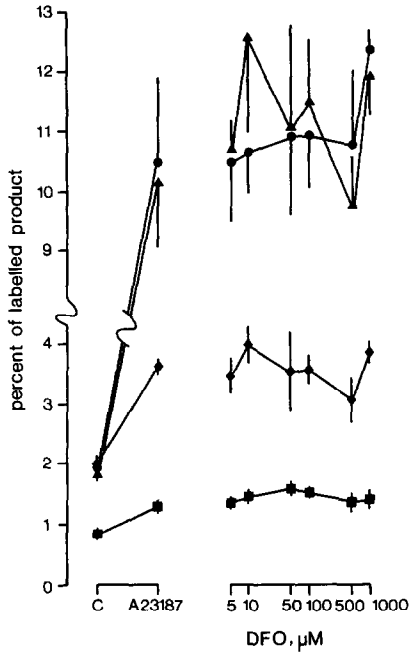

Fig. 2. Effects of desferrioxamine on the release of eicosanoids from rat peritoneal leukocytes stimulated by 1 μM A23187. The various products were measured using a radio-TLC technique and expressed as percentage of total arachidonate converted to each product (▲ = LTB_4, ● = 5-HETE, ◆ = TXB_2, ■ = PGE_2, assignments based on cochromatography of authentic standards and profiling by RIA). Note the breaks of scale and the change in calibration. Results show mean SEM for three tests at each concentration. C = control, DFO = desferrioxamine.

enhanced from the control value of 0.17 ± 0.01 ng/mg/15 min by either desferrioxamine or adrenaline under similar conditions. Results on the last three eicosanoids were all obtained using frozen/thawed caecal fragments.

The ability of desferrioxamine to enhance prostacyclin generation in this system was not shared by iron-loaded desferrioxamine (Table 1, Fig. 1). The complex was essentially inactive, except when tested at 5 mM in frozen/thawed tissues: under these conditions, there was partial inhibition of prostacyclin generation. Its effect on the generation of other eicosanoids was not tested. Ferric ions alone (as ferric chloride) did not affect prostacyclin generation in the concentration range 0.005–5 mM (data not shown).

We also tested the effect of a wide range of desferrioxamine concentrations (0.005–0.5 mM) on A23187-induced and FMLP-induced eicosanoid production by rat peritoneal leukocytes (Fig. 2, Table 2). These cells generate large amounts of 5-lipoxygenase products (LTB_4 and 5-HETE), with smaller quantities of TXB_2 and much less PGE_2 and 6-keto $PGF_{1\alpha}$ (prostacyclin) as evidenced by radioimmunoassay of the metabolites released from ionophore-treated cells, or after radio-chromatography following incubation of the cells with [^{14}C]-arachidonate. None of the concentrations of desferrioxamine affected the ability of the peritoneal leukocytes to convert labelled arachidonate to eicosanoids after stimulating the cells with A23187 (Fig. 2).

However, when release of eicosanoids was stimulated by activating endogenous phospholipase(s) on incubation of the cells with either A23187 or the chemotactic tripeptide, FMLP, we found that the higher concentrations of desferrioxamine caused significant, though erratic, stimulation of LTB_4 and, in the case of the FMLP-stimulated cells, of TXB_2 generation (Table 2). These results were obtained by radioimmunoassay, and the cells were not exposed to exogenous arachidonate. Iron-loaded desferrioxamine and ferric chloride were not tested under these conditions.

DISCUSSION

Desferrioxamine or ferrioxamine at concentrations up to 500 μM had only small effects on caecal and leukocyte eicosanoid production; higher concentrations augmented eicosanoid release in some of the experimental systems. None of the effects involved marked inhibition of eicosanoid output, making it very improbable that the recognised anti-inflammatory effects of desferrioxamine *in vivo* owe anything to inhibition of eicosanoid release.

Subcutaneous administration of 100 mg/kg body weight of desferrioxamine over 24 hr in humans gives maximum blood concentrations of about 18 μM; intravenous injection of 10 mg/kg produced circulating desferrioxamine concentrations of 80–130 μM, falling rapidly to ≤10 μM 30 min later [20]. These concentrations are lower than those which were effective in our experiments.

The stimulatory effect of the unphysiologically high desferrioxamine concentrations on prostanoid

Table 2. Failure of desferrioxamine to inhibit 5-lipoxygenase and cyclo-oxygenase in rat leukocytes stimulated by A23187 and FMLP

Condition	Product formation, ng/5 × 10^6 cells	
	LTB_4	TXB_2
Basal	0.3 ± 0.2	0.1 ± 0.1
A23187, 10^{-6} M	154.8 ± 9.8	27.6 ± 1.2
+DFO, 5 × 10^{-6} M	177.6 ± 17.9	22.9 ± 1.7*
+DFO, 10^{-5} M	197.6 ± 15.0	26.5 ± 0.4
+DFO, 5 × 10^{-5} M	208.4 ± 14.2*	27.3 ± 2.1
+DFO, 10^{-4} M	151.2 ± 23.8	24.8 ± 2.4
+DFO, 5 × 10^{-4} M	196.8 ± 14.2	23.3 ± 1.9
Basal	0	1.2 ± 0.1
FMLP, 10^{-5} M	13.5 ± 1.5	8.4 ± 0.9
+DFO, 5 × 10^{-6} M	14.8 ± 1.1	9.4 ± 0.9
+DFO, 10^{-5} M	16.3 ± 1.0	10.1 ± 1.1
+DFO, 5 × 10^{-5} M	19.6 ± 1.5*	10.8 ± 0.9
+DFO, 10^{-4} M	17.9 ± 0.8*	11.5 ± 0.3*
+DFO, 5 × 10^{-4} M	25.8 ± 2.9*	9.7 ± 0.7

Values show mean ± SEM of triplicate incubations, each assayed by RIA in duplicate. *Shows significant difference from value obtained for cells stimulated with A23187 or FMLP in absence of desferrioxamine (DFO), $P < 0.05$ by Student's unpaired *t*-test.

production by rat caecal fragments suggests that it may be acting by increasing cyclo-oxygenase activity, because amounts were increased of all three cyclooxygenase products that were measured. Perhaps desferrioxamine prevents self-inactivation of the enzyme by scavenging the [O_x] species [21–23], since high concentrations of desferrioxamine are known to have a radical-scavenging effect [12]. However, the inability of ferrioxamine to produce the same action suggests that iron-chelation may be involved.

Our results also suggest that desferrioxamine is able to enter cells within the caecal tissue during the 60 + 15 min incubation periods. Despite the stimulation of prostanoids, LTB_4 production was unaffected indicating that desferrioxamine had no action on lipoxygenase in this system. This fact might argue against an alternative explanation for desferrioxamine's action at high concentration, namely that it increases the release of arachidonic acid within the tissue. However, it is known that factors additional to free arachidonate, such as Ca^{2+} and ATP, are necessary to activate the 5-lipoxygenase enzyme [24], and these conditions may not be satisfied merely by adding high concentrations of desferrioxamine. Although we do not know the extent to which release of arachidonate within the caecal fragments occurs spontaneously in freshly prepared tissues, it seems reasonable to suppose that it may be rate limiting for the formation of eicosanoids. Further, it may be enhanced in the tissues subjected to freeze/thawing (therefore making this a less "physiological" model system), thus explaining the larger eicosanoid levels observed in these specimens (see Table 1).

In contrast to the results for caecal fragments, some evidence was obtained showing that desferrioxamine can enhance leukotriene generation by rat leukocytes, at least when the experiments were performed in the absence of exogenous arachidonate (Table 2). Overall, there was again no evidence to suggest that desferrioxamine has any meaningful capacity to inhibit 5-lipoxygenase, as previously implied [15].

Thus taken overall, our results show clearly that it is highly unlikely that the anti-inflammatory actions of desferrioxamine *in vivo* can be explained by direct alteration of eicosanoid metabolism, at therapeutically safe concentrations.

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