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The iron chelators desferrioxamine and 1-alkyl-2-methyl-3-hydroxypyrid-4-ones inhibit vascular prostacyclin synthesis *in vitro*

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The iron chelators desferrioxamine (DFO), 1,2-dimethyl(L1)-, 1-ethyl-2-methyl(L1NEt)- and 1-propyl-2-methyl(L1NPr)-3-hydroxypyrid-4-ones inhibited rat aortic prostacyclin (PGI_2) synthesis *in vitro* (rank order of potency: DFO > L1 > L1NEt > L1NPr) when stimulated with adrenaline, arachidonate and the Ca^{2+} ionophore A23187. The inhibitory action of the chelators was blocked by Fe^{3+} and Al^{3+} and reversed by washing and H_2O_2, but not by ascorbate. These data suggest that iron chelators inhibit prostanoid synthesis in intact tissue through the removal or binding of Fe^{3+} linked to cyclo-oxygenase. These iron chelators may be of therapeutic value in the treatment of inflammatory and other diseases via two mechanisms: (1) the inhibition of pro-inflammatory prostanoid synthesis and (2) the inhibition of toxic-free-radical generation by cyclo-oxygenase.

INTRODUCTION

Cyclo-oxygenase (EC 1.14.99.1) is a holoenzyme which catalyses the oxygenation and cyclization of certain fatty acids (Samuelsson, 1965) to yield the endoperoxide prostanoid precursor prostaglandin G_2 (PGG_2; Hamberg & Samuelsson 1973; Nugteren & Hazelhof, 1973). Purified cyclo-oxygenase contains haem (Kulmacz & Lands, 1984), which acts as a catalytic centre for the generation of oxygen co-factors. Little is known about the effects of metabolic iron imbalance on prostanoid synthesis; however, since it is known that (1) rheumatoid synovial membrane contains high concentrations of iron (Muirden *et al.*, 1966) and (2) prostanoids are mediators of inflammation (Lewis, 1983), it is possible that there is an aetiological link between elevated iron and prostanoid synthesis in rheumatoid arthritis and other inflammatory diseases. In this context it has been suggested that iron chelators may prove beneficial in the treatment of such diseases through the removal of iron and diminution of its ability to catalyse production of oxy radicals (Gutteridge *et al.*, 1979; Halliwell & Gutteridge, 1984) and also that the design of site-specific chelators may be required to inhibit the various iron centres associated with the formation of toxic oxygen-activated products (Kontoghiorghes, 1987*b*).

In order to investigate further the relationship between iron and prostanoid synthesis, the effects of four potent chelators of iron, namely desferrioxamine, 1,2-dimethyl-3-hydroxypyrid-4-one (L1), 1-ethyl-2-methyl-3-hydroxypyrid-4-one (L1NEt) and 1-propyl-2-methyl-3-hydroxypyrid-4-one (L1NPr), on vascular prostacyclin (PGI_2) synthesis by the rat aorta were studied by using established '*in vitro*' systems (Jeremy *et al.*, 1985*a*,*b*, 1986*a*). Possible sites of iron-chelator action were investigated by using a range of stimulators of prostanoid synthesis, namely adrenaline, arachidonate and the Ca^{2+} ionophore A23187 (Jeremy & Dandona, 1986*a*; Jeremy *et al.*, 1985*a*, 1986*a*). The action of the chelators in the presence of both iron and aluminium was also investigated in order to examine possible chelator–metal-complex-mediated interactions. Furthermore, since it was suggested that molecules with antioxidant or radical-trapping properties may act as reversible non-competitive inhibitors of cyclo-oxygenase (Warso & Lands, 1983) and that certain iron chelators are potent antioxidants (Kontoghiorghes *et al.*, 1986), the effects of H_2O_2 and ascorbic acid, as well as the interactions of these two compounds with the chelators, were also investigated.

MATERIALS AND METHODS

The chelators L1, L1NEt and L1NPr were prepared as previously described (Kontoghiorghes & Sheppard, 1987). Adrenaline bitartrate, arachidonic acid, calcium ionophore A23187, H_2O_2 (30%, v/v) and ascorbic acid were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Tissue preparation

Male Sprague–Dawley rats (300 g) were decapitated and their aortae rapidly excised and placed in Krebs–Ringer bicarbonate buffer [KRB; pregassed with O_2/CO_2 (19:1) to pH 7.35–7.4]. KRB consisted of (mmol·l^{-1}: NaCl, 118.6; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.19; Mg_2SO_4, 1.19; $NaHCO_3$, 2.46 and glucose, 5.56), made up in double-distilled water collected in polypropylene flasks. Tissue was then prepared for the assessment of PGI_2 synthesis as previously described

Abbreviations used: DFO, desferrioxamine; L1, L1NEt and L1NPr, 1,2-dimethyl-, 1-ethyl-2-methyl- and 1-propyl-2-methyl-3-hydroxypyrid-4-one respectively; PGG_2, prostaglandin G_2; PGI_2, prostaglandin I_2; KRB. Krebs–Ringer bicarbonate buffer (for composition, see the text); 6-oxo-$PGF_{1\alpha}$, 6-oxoprostaglandin $F_{1\alpha}$ IC_{50}, concentration of chelator at which agonist stimulation was inhibited by 50%; PGH_2, prostaglandin H_2.

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(Jeremy *et al.*, 1985*a*,*b*, 1986*a*). Aortae were cleared of adventitia and cut into 1 mm rings with a scalpel blade on a Teflon block. Tissues were pooled and randomized in KRB and incubated at 37 °C for 6 h in a shaking water bath to allow preparation-elicited prostanoid synthesis to subside (Jeremy *et al.*, 1984, 1985*a*, 1986*a*) before incubation with chelators and agonists. In all experiments, polypropylene flasks and tubes were used to obviate glass-derived contamination of buffers with iron and other tervalent cations.

Incubation of aortic tissue with agonists and chelators

After 6 h preincubation, four aortic rings were placed in 1 ml of KRB containing chelators of different concentrations (1×10^{-4}–1×10^{-2} mol·l^{-1}), in sextuplicate for each chelator concentration. Two untreated batches (without chelators) were included, one of which was the zero basal group and the other the agonist alone group. The tissues were equilibrated for 60 min at 37 °C, the KRB aspirated and fresh KRB containing the same concentrations of chelators was added. Prostanoid synthesis was then stimulated by the addition of adrenaline (3×10^{-6} mol·l^{-1} final concn.; Jeremy *et al.*, 1985*a*). In order to investigate the enzymic site of action of the chelators, PGI_2 was also stimulated in rat aortae with the Ca^{2+} ionophore A23187 (3×10^{-5} mol·l^{-1}; Jeremy *et al.*, 1986*a*) and arachidonate (3×10^{-5} mol·l^{-1}; Jeremy & Dandona, 1986*a*). After the addition of agonists, the tissues were incubated in a shaking water bath at 37 °C for 1 h. Portions of the supernatants were taken for estimation of 6-oxo-$PGF_{1\alpha}$ (the stable, spontaneous 'hydrolysate' of PGI_2; Moncada & Vane, 1979) by radioimmunoassay (see below). Previous studies have established that, in the present '*in vitro*' system, the functional integrity of the tissue remains intact (namely specific receptor agonist and antagonist responses, receptor-linked calcium channels and receptor-linked protein kinase C and G proteins; Jeremy & Dandona, 1987, 1988; Jeremy *et al.*, 1985*a*,*b*, 1986*a*,*b*). Furthermore, transmission electron microscopy has previously revealed no changes in tissue structure after incubation in the present system (Jeremy & Dandona, 1986*a*,*b*).

In order to test whether the chelators were removing an obligatory metal cation or were exerting a direct action on prostanoid-synthesizing enzymes, tissues were preincubated for 4 h with the chelators, the buffer (containing the same concentrations of chelators) being changed every 30 min. The tissues were then washed in chelator-free KRB made up in deionized double-distilled water, and prostanoid synthesis was stimulated with arachidonate, as described above.

In separate experiments the effects of $FeCl_3$ as well as $AlCl_3$ (up to 1 mmol·l^{-1}) on agonist-stimulated synthesis *de novo* of aortic PGI_2 and interactions of iron and aluminium with chelators on PGI_2 synthesis were also studied by the same method as that described above.

The effect of ascorbic acid and H_2O_2 on aortic PGI_2 synthesis *de novo* was investigated singly in separate experiments as for the action of agonists on prostanoid synthesis (Jeremy *et al.*, 1985*a*,*b*, 1986*a*). The effect of H_2O_2 and ascorbic acid on chelator inhibition of aortic PGI_2 synthesis was subsequently studied as described above.

Radioimmunoassay of 6-oxo-$PGF_{1\alpha}$

Radioimmunoassay procedures and validation of methods have been previously described (Jeremy *et al.*, 1985*a*,*b*, 1986*a*,*b*). Antisera against 6-oxo-$PGF_{1\alpha}$ were purchased from Capell Laboratories (West Chester, PA, U.S.A.). [^{3}H]-6-oxo-$PGF_{1\alpha}$ (120 Ci·mmol^{-1}) was purchased from New England Nuclear (Dreieich, Germany) and unlabelled ligand from Cayman Chemical Co. (Palo Alto, CA, U.S.A.).

RESULTS

Effect of chelators on adrenaline-, ionophore-A23187- and arachidonate-stimulated PGI_2 synthesis

Desferrioxamine, L1, L1NEt and L1NPr inhibited adrenaline-, Ca^{2+}-ionophore- and arachidonate-stimulated PGI_2 synthesis by aortic tissue in concentration-dependent fashion (Figs. 1, 2 and 3). The quantities of PGI_2 released in response to agonists is given in the Figure legends. The rank order of potency was desferrioxamine > L1 > L1NEt > L1NPr (for IC_{50} values, see the Figure legends). At physiological pH, desferrioxamine binds iron at 1:1 and the 1-alkyl-2-methyl-3-hydroxypyrid-4-ones at 3:1 (chelator:iron) molar ratio respectively (Kontoghiorghes *et al.*, 1987*a*). Thus the greater potency of desferrioxamine over L1, L1NEt and L1NPr is consistent with its higher binding capacity over the other chelators. Furthermore, the three 1-alkyl-2-methyl-3-hydroxypyrid-4-ones have similar iron-binding constants ($\beta_3 = 10^{36}$), but differ in the n-octanol/water partition coefficients ($K_{par.}$) as follows: L1, 0.19; L1NEt,

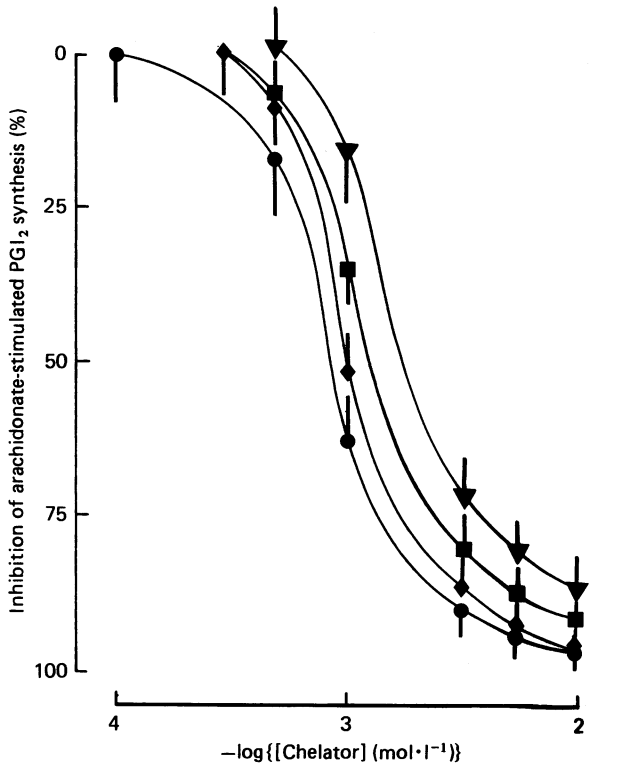

Fig. 1. Effect of iron chelators on arachidonate (3×10^{-5} mol·l^{-1} final concn.)-stimulated rat aortic PGI_2 synthesis (190 pg·min^{-1}·mg^{-1})

IC_{50} values (mol·l^{-1}) were as follows: DFO (●), 8.8×10^{-4}; L1 (◆), 1×10^{-3}; L1NEt (■), 1.3×10^{-3}; L1NPr (▼), 1.8×10^{-3}. Each point represents the mean ± S.D. ($n = 6$).

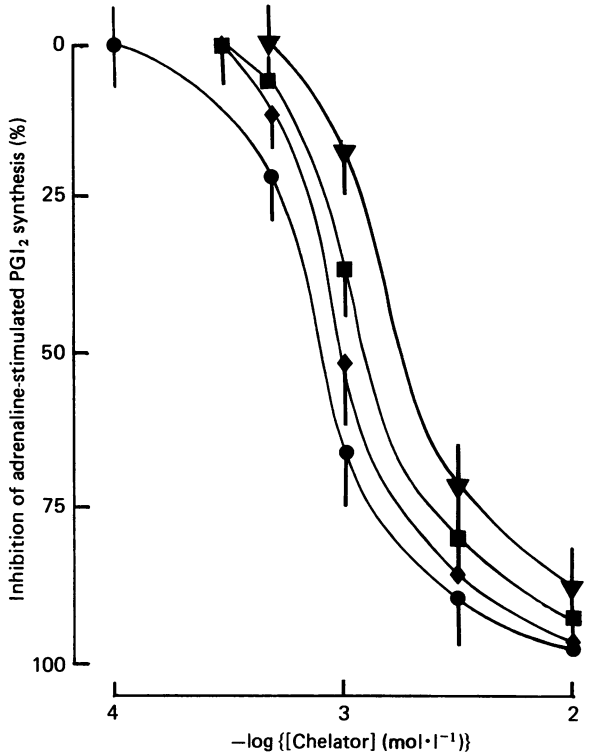

Fig. 2. Effect of iron chelators on adrenaline (3×10^{-5} mol·l^{-1} final concn.)-stimulated rat aortic PGI_2 synthesis (160 pg·min^{-1}·mg^{-1})

IC_{50} values (mol·l^{-1}) were as follows: desferrioxamine (●), 7.8×10^{-4}; L1 (◆), 1×10^{-3}; L1NEt (■), 1.3×10^{-3}; L1NPr (▼), 1.9×10^{-3}. Each point represents the mean ± S.D. ($n = 6$).

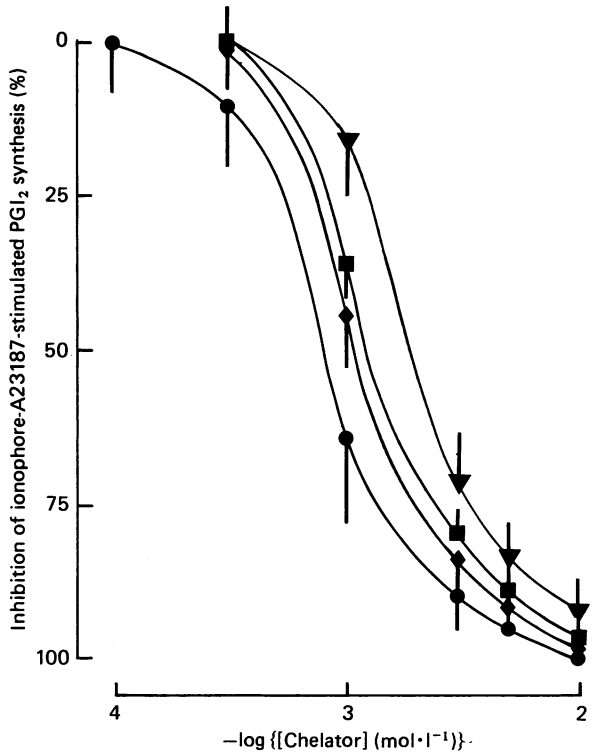

Fig. 3. Effect of iron chelators on A23187 (3×10^{-5} mol·l^{-1} final concn.)-stimulated rat aortic PGI_2 synthesis (165 pg·min^{-1}·mg^{-1})

IC_{50} values (mol·l^{-1}) were as follows: desferrioxamine (●), 8×10^{-4}; L1 (◆), 1.2×10^{-3}; L1NEt (■), 1.4×10^{-3}; L1NPr (▼), 2×10^{-3}. Each point represents the mean ± S.D. ($n = 6$).

0.97; L1NPr, 3.16 (Kontoghiorghes & Sheppard, 1987). Thus the relative order of potency of these three chelators on the inhibition of aortic PGI_2 synthesis suggests that the inhibitory action of the chelators is related to their hydrophilicity. It is worth mentioning that the $K_{par.}$ of their iron complexes are also of the same order, and that DFO and its iron complex are even more hydrophilic, as follows: DFO, 0.02; DFO–Fe, 0.02; $[L1]_3$–Fe, 0.24; $[L1NEt]_3$–Fe,0.52; and $[L1NPr]_3$–Fe, 4.12 (Kontoghiorghes & Sheppard 1987; Kontoghiorghes, 1988).

The equipotent inhibition of the chelators on arachidonate-stimulated PGI_2 synthesis (Fig. 1) when compared with A23187- (Fig. 3) and adrenaline- (Fig. 2) stimulated PGI_2 synthesis indicates that the chelators are acting on cyclo-oxygenase rather than on phospholipase A_2 (Jeremy *et al.*, 1986*a*). The rationale for this is that adrenaline and A23187 stimulate aortic PGI_2 synthesis through initiating Ca^{2+} influx (Jeremy & Dandona, 1986*a*; Jeremy *et al.*, 1985*a*, 1986*a*), thereby activating phospholipase A_2, the enzyme which liberates endogenous arachidonate from phospholipid stores (Irvine, 1982). The addition of exogenous arachidonate in the present system, therefore, by-passes phospholipase A_2 activation and as such is an index of cyclo-oxygenase.

Ionized calcium was unaffected by the chelators at the concentrations used in the present study [ionized calcium was assessed using an ICA (Copenhagen, Denmark) ionized calcium analyser]. Furthermore, the chelators have low affinity for Ca^{2+} (Kontoghiorghes *et al.*, 1987*a*), indicating that the present inhibitory action of the chelators on aortic PGI_2 synthesis is not due to calcium chelation or inhibition of phospholipase A_2. Furthermore, there is no evidence that phospholipase A_2 is dependent on iron or another tervalent cation.

Reversibility of chelator inhibition of aortic PGI_2 synthesis by Fe^{3+}, Al^{3+} and washing

Both Fe^{3+} and Al^{3+} (both 1×10^{-3} mol·l^{-1} final concn.) blocked the inhibitory action of the chelators (DFO, 1×10^{-3} mol·l^{-1}; L1, L1NEt and L1NPr, 3×10^{-3} mol·l^{-1}) on arachidonate-stimulated PGI_2 synthesis (Fig. 4). This effect is attributable to the fact that these exogenous metal cations complexed with the chelators, thereby either inhibiting chelator uptake by the tissue and/or negating chelating actions on endogenous aortic cations. Fe^{3+} and Al^{3+} alone, at 1 mmol·l^{-1}, were without effect on '*de novo*' or agonist-stimulated PGI_2 synthesis (results not shown).

The inhibitory action of the chelators was reversed by washing with chelator-free KRB after a 4 h preincubation of the tissues with the chelators (Fig. 5). These data indicate that the chelators are not toxic to the present systems, which are dependent on tissue integrity (Jeremy *et al.*, 1984, 1985*a*; Jeremy & Dandona, 1986*a*).

Effect of H_2O_2 and ascorbic acid

H_2O_2 was a potent stimulator of synthesis of rat aortic PGI_2 synthesis *de novo* (Fig. 6), whereas ascorbic acid

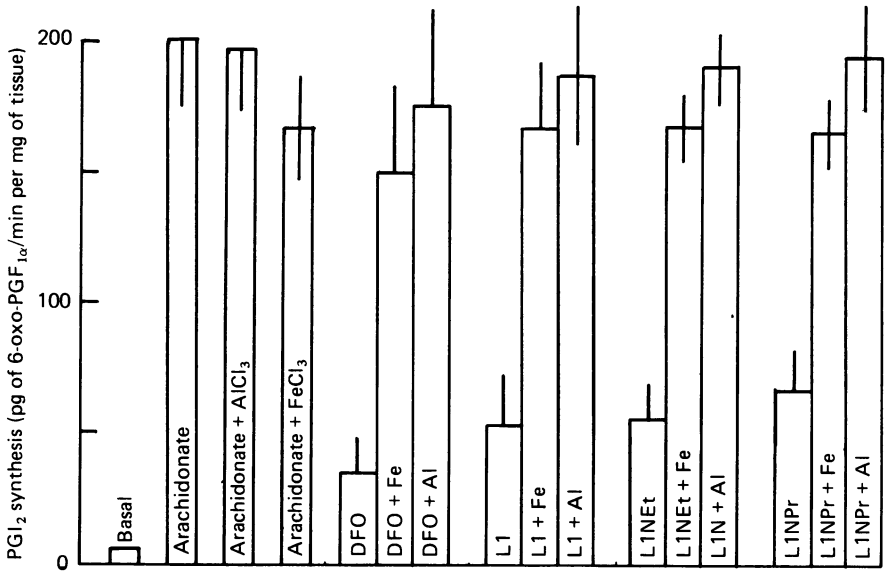

Fig. 4. Effect of $FeCl_3$ and $AlCl_3$ (both 1×10^{-3} mol·l^{-1} final concn.) on chelator-inhibited (DFO, 1×10^{-3} mol·l^{-1} final concn.; L1, L1NEt and L1NPr, 3×10^{-3} mol·l^{-1} final concn.) rat aortic PGI_2 synthesis [arachidonate (3×10^{-5} mol·l^{-1})-stimulated]

Each bar represents the mean ± S.D. ($n = 6$).

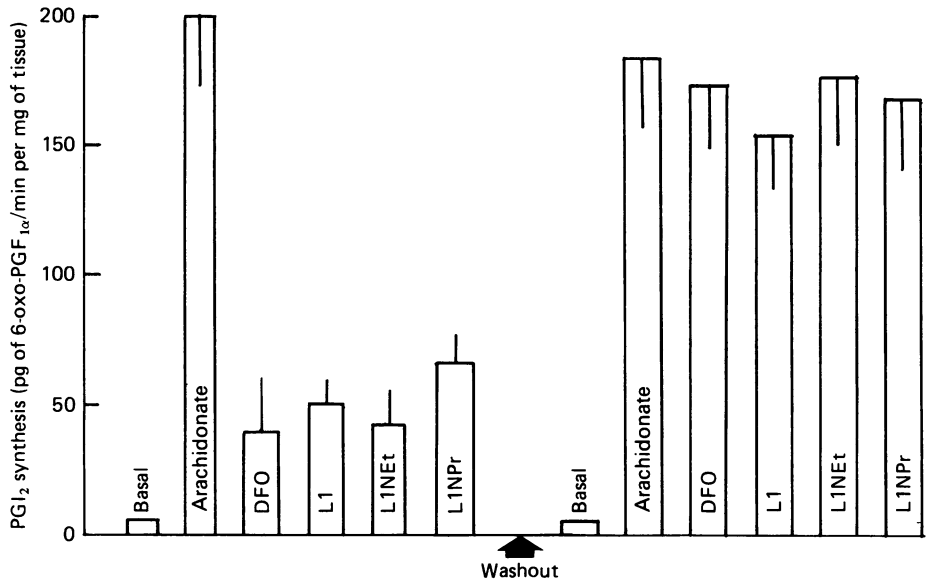

Fig. 5. Effect of washing with chelator-free KRB (4 × 15 min) on rat aortic PGI_2 synthesis after 4 h incubation with iron chelators (all at 3×10^{-3} mol·l^{-1})

PGI_2 synthesis was stimulated with arachidonate (3×10^{-5} mol·l^{-1}). Each bar represents the mean ± S.D. ($n = 6$).

was slightly stimulatory, but to a far lesser extent than H_2O_2 (Fig. 6). The inhibitory actions of the chelators on adrenaline-stimulated PGI_2 by rat aorta were reversed by the presence of H_2O_2 (Fig. 7), whereas ascorbic acid was without effect (results not shown).

DISCUSSION

The present study demonstrates that, in an intact tissue system, the iron chelators DFO, L1, L1NEt and L1NPr reversibly inhibit vascular cyclo-oxygenase activity. Since cyclo-oxygenase contains haem (Kulmacz & Lands, 1984) and there is no evidence that other tervalent cations influence cyclo-oxygenase activity, this inhibitory action is probably the result of iron removal or the inhibition of an iron-dependent centre related to cyclo-oxygenase.

The reversibility of the chelator action by washing or by metal-complex formation suggests that different pools of iron (Kontoghiorghes, 1987*b*), may be involved in mediating cyclo-oxygenase activity. Thus a readily chelated low-M_r pool of iron (associated with the enzymic turnover of cyclo-oxygenase) may initially be affected, resulting in inhibition of PGI_2 synthesis. However, after

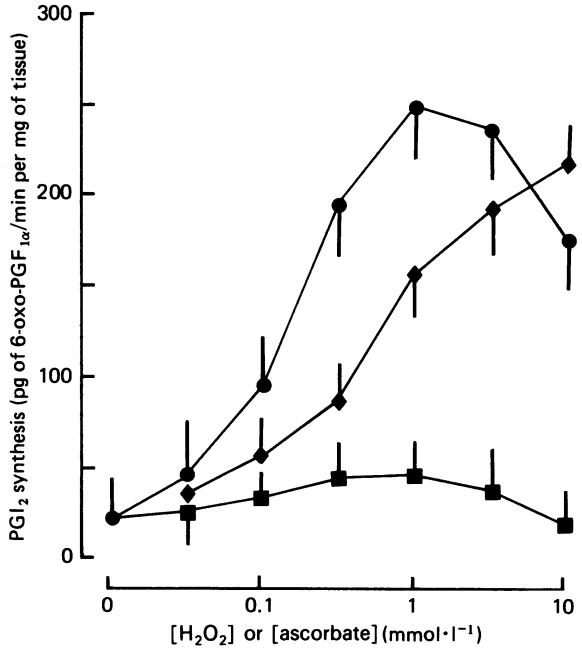

Fig. 6. Effect of H_2O_2 (●), H_2O_2 + desferrioxamine (1×10^{-3} mol·l^{-1}) (◆) and ascorbic acid (■) on rat aortic PGI_2 synthesis *de novo*

Each point represents the mean ± S.D. ($n = 6$).

removal of the chelator by washing, a storage-iron pool (perhaps ferritin), which is not readily chelated by the present chelators may replenish the low-M_r iron pool associated with cyclo-oxygenase activity. This latter suggestion is consistent with the observation that iron associated with ferritin is slowly removed with iron chelators (Kontoghiorghes, 1986*a*) and it can only be totally removed with strong chelators such as DFO and L1 after prolonged incubation of up to 24 days (Kontoghiorghes, 1987*a*). Alternatively, the chelators could inhibit cyclo-oxygenase by binding or oxidizing its haem iron. Similar mechanisms were suggested previously in the inhibition of a non-haem iron-containing enzyme, namely ribonucleotide reductase (Hoffbrand *et al.*, 1976) or the oxidation of haemoglobin by DFO (Kontoghiorghes, 1987*c*).

The stimulatory effect of H_2O_2 on aortic PGI_2 synthesis *de novo* is consistent with previous reports on H_2O_2 and prostanoid synthesis in other tissues (Polgar & Taylor, 1980). By contrast, the lack of effect of ascorbate on aortic PGI_2 synthesis indicates that the reducing properties of this vitamin could not reverse the inhibitory action of the chelators, which could be mediated through oxidation of ferrous iron (Kontoghiorghes *et al.*, 1986; Kontoghiorghes, 1987*b*).

The reversal of the chelator-induced inhibition of prostacyclin synthesis by H_2O_2 further suggests that the stimulatory effect of H_2O_2 on aortic PGI_2 synthesis may be mediated by oxygen-activated products of iron catalysis. Under normal physiological conditions, cyclo-oxygenase catalyses the hydroperoxidation of arachidonate to yield the endoperoxide PGG_2 (Warso & Lands, 1983; Bakhle, 1983), which is then converted into PGH_2 by peroxidase action, thought to be cyclo-oxygenase itself (Lands, 1979) and then into PGI_2 by a specific synthase (Moncada & Vane, 1979). It has also been demonstrated that lipid hydroperoxides are obligatory for cyclo-oxygenase activity (Warso & Lands, 1983) and that cyclo-oxygenase-associated iron may play a role in generating other radicals (Mason *et al.*, 1980).

Cyclo-oxygenase also possesses the potential to generate other oxygen-activated products which could give rise to oxygen free radicals ($O_2^{-\cdot}$, $OH^{\cdot}$ and $HO_2^{\cdot}$) as well as peroxidation products. These lipid peroxides and other free radicals have been widely implicated in the pathophysiology of inflammation and other diseases (Halliwell & Gutteridge, 1984; Slater, 1984; Editorial,

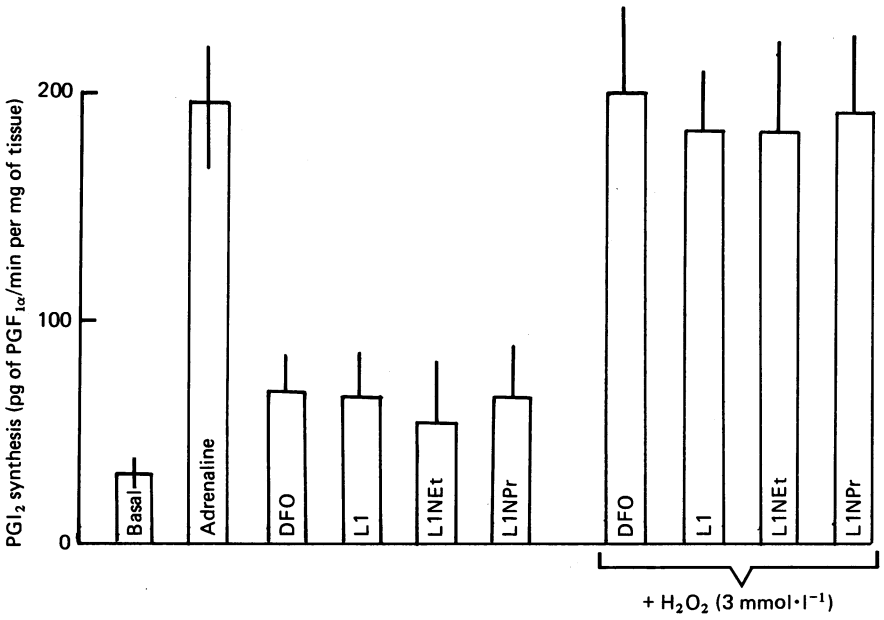

Fig. 7. Effect of H_2O_2 (3×10^{-3} mol·l^{-1}) on chelator-inhibited rat aortic PGI_2 synthesis [adrenaline (3×10^{-5} mol·l^{-1})-stimulated]

Each bar represents the mean ± S.D. ($n = 6$).

1985). Thus the inhibition of cyclo-oxygenase by iron chelators may be of therapeutic value in the treatment of inflammatory disease via two mechanisms: firstly through inhibition of pro-inflammatory prostanoid synthesis and secondly through inhibition of toxic-free-radical generation by cyclo-oxygenase. These proposals warrant further investigation.

The present study also demonstrates that the 1-alkyl-2-methyl-3-hydroxypyrid-4-one compounds are almost as potent as DFO in inhibiting prostanoid synthesis. These compounds were developed for oral administration in the treatment of iron overload and other diseases of iron imbalance and toxicity (Kontoghiorghes, 1985, 1986*b*; Kontoghiorghes *et al.*, 1987*b*,*c*). The relative potency of inhibition of aortic PGI_2 synthesis by these chelators accords with their relative hydrophilicities (DFO > L1 > L1NEt > L1NPr; Kontoghiorghes & Sheppard, 1987; Kontoghiorghes, 1988). It is therefore proposed that the site of inhibition of the chelators in the present study (namely cyclo-oxygenase) is a hydrophilic cellular component. Furthermore, the differential hydrophilicity of iron chelators may be of relevance to rationalizing not only the possible uses of these drugs in inflammatory and other diseases, but also any side effects that may arise. It is also relevant that 1,2-dimethyl-3-hydroxypyrid-4-one has so far been shown to be free from side effects in the treatment of iron overload in thalassaemia and myelodysplasia patients (Kontoghiorghes *et al.*, 1987*b*,*c*).

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