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Oxidation of uroporphyrinogens by hydroxyl radicals

Evidence for nonporphyrin products as potential inhibitors of uroporphyrinogen decarboxylase

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A hydroxyl radical-generating system, hypoxanthine/xanthine oxidase/Fe-EDTA, oxidises uroporphyrinogens to uroporphyrins and to more polar nonporphyrin products. The evidence suggests that the nonporphyrin products have inhibitory activity towards mouse liver uroporphyrinogen decarboxylase which could explain some forms of human and experimental porphyrias.

Uroporphyrinogen; Hydroxyl radical; Uroporphyrinogen decarboxylase

1. INTRODUCTION

Human sporadic porphyria cutanea tarda and porphyria caused by some chemicals in man and rodents, are characterised by inhibition of hepatic uroporphyrinogen decarboxylase [1,2]. The mechanism is unknown but recently favoured theories invoke reactive oxygen species generated from an uncoupled microsomal system [3-8]. The oxidation of uroporphyrinogen to uroporphyrin occurs with chick embryo liver microsomes in the presence of NADPH and 3,4,3′,4′-tetrachlorobiphenyl [6,8] and could account for the accumulation of uroporphyrin in some experimental systems [3,9]. However, it has been proposed that in mammals treated with porphyrogenic chemicals a modified uroporphyrinogen or uroporphyrin may be formed which subsequently inhibits the decarboxylase [5,8,10]. As a model we have examined the effects of the hypoxanthine/xanthine oxidase/Fe-EDTA hydroxyl radical-generating system [11-13] on uroporphyrinogens and determined whether products, besides uroporphyrin, were formed that inhibited uroporphyrinogen decarboxylase.

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2. EXPERIMENTAL

Specialized chemicals were purchased from the following sources: desferrioxamine mesylate, Ciba-Geigy, Switzerland; uroporphyrins I and III, pentacarboxyporphyrin I and hydroxymethylbilane, Porphyrin Products, USA; mouse liver catalase (5400 U/mg), bovine kidney superoxide dismutase (10000 U/mg), butter milk xanthine oxidase (0.9 U/mg) and hypoxanthine, Sigma, England. [^{14}C]Uroporphyrin (a mixture of the I and III isomers) was as previously described [14]. Porphyrinogens were prepared by Na-amalgam reduction of porphyrins [14]. Solutions were prepared with double glass-distilled water which was further purified though a Millipore deionizer.

Basic incubation mixtures contained 0.5 mM hypoxanthine and 0.12 M sodium phosphate buffer (pH 7.4) in a final volume of 2.5 ml. When appropriate a Fe^{3+}-EDTA mixture was added (final concentrations 0.05 and 0.15 mM, respectively) and xanthine oxidase (0.2 U/ml). Incubations were initiated by the addition of uroporphyrinogen (final concentration 3.3 μM) and continued at 22°C in the dark. Absorption spectra of incubations were determined at the end of experiments and sometimes at more frequent intervals. Enough 0.1 M iodine solution in KI was then added to oxidise any remaining uroporphyrinogen until there was no further increase in the Soret peak. Fluorescence spectroscopy was performed as described by Granick et al. [15].

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Reverse-phase HPLC of uroporphyrin was carried out as in [16]. Incubations with [^{14}C]uroporphyrinogen were treated as above except that fractions were collected after HPLC and radioactivity contents then determined. Inhibition of uroporphyrinogen decarboxylase activity after preincubation with products of uroporphyrinogen oxidation was estimated using pentacarboxyporphyrinogen I as substrate and 40000×*g* C57BL/10ScSn mouse liver supernatant as the enzyme source [16,17].

3. RESULTS

3.1. *Oxidation of uroporphyrinogens*

In the presence of hypoxanthine/xanthine oxidase/Fe-EDTA, uroporphyrinogen III was rapidly oxidised to uroporphyrin (fig.1). Without iron or xanthine oxidase the conversion of uroporphyrinogen to uroporphyrin was much reduced indicating a probable involvement of hydroxyl radical. The same conclusion was suggested by the protective effects of catalase, mannitol and desferrioxamine although superoxide dismutase did not protect and instead consistently gave enhanced porphyrin formation (table 1). Both uroporphyrinogens I and III were oxidised. In addition, oxidation to nonporphyrin products occurred, since after careful reoxidation of remaining porphyrinogen with iodine, the final yield of uroporphyrin recovered was significantly lower in incubations with xanthine oxidase/Fe-EDTA whether this was estimated from the absorption spectrum or by HPLC (table 2). No major additional peaks during HPLC could be detected when monitoring for porphyrins at 400 nm to account for this loss. Both catalase and superoxide dismutase partially protected from the reduction in yield (not shown). Fluorescence spectroscopy also illustrated a reduced yield of recovered porphyrin after treatment of uroporphyrinogen III for 30 min with hydroxyl radical, i.e. relative fluorescence, control 100; xanthine oxidase/Fe-EDTA, 67. Uroporphyrins were only slowly affected by hydroxyl radical (<7% in 30 min) when compared with porphyrinogens whether this was assessed by absorption spectroscopy (fig.1), fluorescence spectroscopy or HPLC.

Hydroxymethylbilane was also oxidised to uroporphyrin I at an increased rate (150% of control) by incubation with xanthine oxidase/Fe-EDTA with a decreased yield of recovered porphyrin (75%) as estimated by absorption spectroscopy and HPLC. Whether hydroxymethylbilane itself was attacked or the uroporphyrinogen formed from it was not determined.

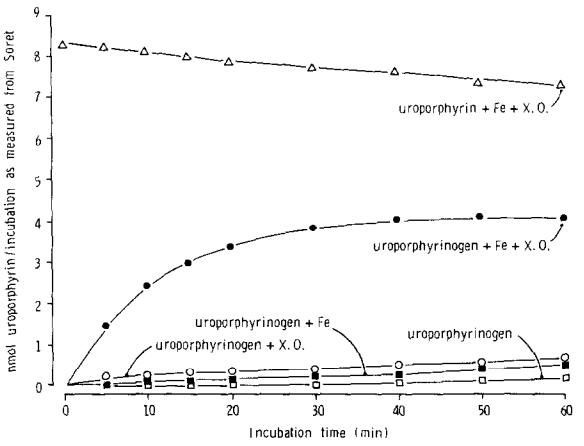

Fig.1. Oxidation of uroporphyrinogen III (3.3 μM) in the presence of the hypoxanthine/xanthine oxidase (X.O)/Fe-EDTA hydroxyl radical-generating system.

3.2. *Oxidation of [^{14}C]uroporphyrinogen*

[^{14}C]Uroporphyrinogen was oxidised by xanthine oxidase/Fe-EDTA and the recovered products were examined by estimating radioactivity contents of fractions following HPLC (fig.2). The radioactivity associated with uroporphyrin was reduced to 64% of control whereas that of fractions 1-7, corresponding to polar products, was increased >5-fold (fig.2A,D). No additional peaks absorbing at 400 nm were observed to account for

Table 1

Effects of scavengers on uroporphyrinogen III oxidation to uroporphyrin

Conditions	nmol uroporphyrin formed/30 min	% protection
Basic	4.2	–
+ superoxide dismutase (100 U/ml)	4.9	−17
+ catalase (200 U/ml)[a]	1.6	62
+ desferrioxamine (0.5 mM)	2.5	40
+ mannitol (10 mM)	2.6	38

[a] Greater quantities of catalase afforded only slight additional protection

Incubations contained hypoxanthine, xanthine oxidase, Fe-EDTA and uroporphyrinogen III as described in section 2.

Table 2

Oxidation of uroporphyrinogens I and III and recovery of uroporphyrins

Conditions[a]	Uroporphyrin formed in 30 min[b] (nmol)		Recovery of uroporphyrin[c]			
			By Soret (nmol)		By HPLC (relative)	
	I	III	I	III	I	III
Control	0.1	0.1	7.3	6.7	100	100
Fe-EDTA	0.5	0.2	6.8	6.0	99	106
X.O.	1.6	0.4	5.6	4.8	67	71
X.O. + Fe-EDTA	4.4	3.9	4.5	3.7	55	46

[a] Conditions were as described in section 2
[b] Uroporphyrin formation was estimated from the Soret peak in the absorption spectrum
[c] Estimated after gentle oxidation with iodine solution of any uroporphyrinogen remaining

this increased radioactivity (fig.2E,F) again suggesting that the polar oxidation products did not contain the porphyrin aromatic system. Catalase and superoxide dismutase decreased radioactivity present in fractions 1–7 by 90 and 75% respectively, when included in xanthine oxidase/Fe-EDTA incubations.

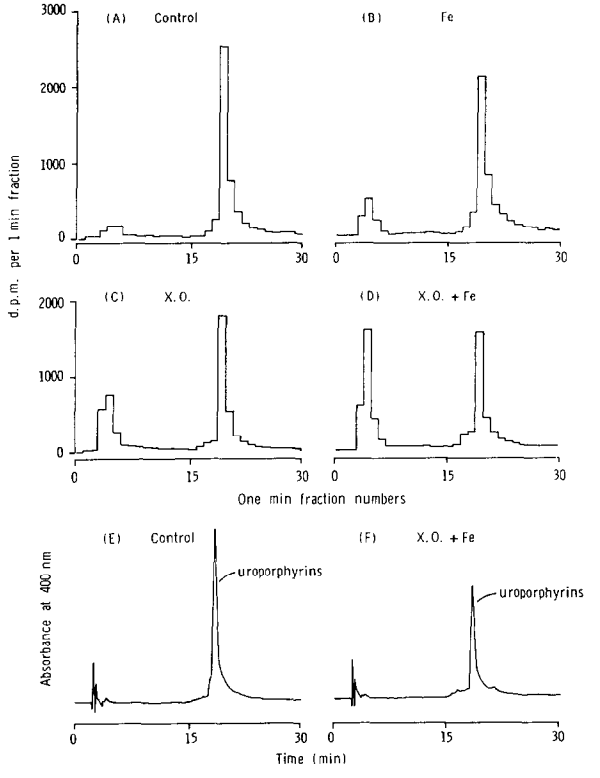

Fig.2. HPLC of incubations of [^{14}C]uroporphyrinogen with hypoxanthine/xanthine oxidase (X.O.)/Fe-EDTA.

3.3. *Incubation of uroporphyrinogen decarboxylase*

Aliquots from incubations of uroporphyrinogen III with the hydroxyl radical-producing systems hypoxanthine/xanthine oxidase/Fe-EDTA and H_2O_2/Fe-EDTA caused a modest but consistent inhibition of uroporphyrinogen decarboxylase ac-

Table 3

Inhibition of uroporphyrinogen decarboxylase

Conditions	Relative decarboxylase activity
Expt A	
X.O. + Fe-EDTA	100
X.O. + Fe-EDTA + uroporphyrin III	95
X.O. + Fe-EDTA + uroporphyrinogen III	62
Expt B	
H_2O_2 + Fe-EDTA	100
H_2O_2 + Fe-EDTA-uroporphyrin III	73
H_2O_2 + Fe-EDTA-uroporphyrinogen III	51

Incubations with xanthine oxidase (X.O.) + Fe^{3+}-EDTA (expt A) were as described in section 2 whereas those with H_2O_2 (0.3 mM) contained Fe^{2+}-EDTA (expt B). After 30 min at 22°C these incubations were stopped with iodine and an aliquot (0.2 ml) incubated with C57BL/10ScSn mouse liver 40000 × *g* supernatant for 10 min at 37°C [20]. Residual uroporphyrinogen decarboxylase activity was then determined using pentacarboxyporphyrinogen I as the substrate [20]. The final concentration of uroporphyrin- or uroporphyrinogen-derived matieral in a decarboxylase assay was 0.66 μM

tivity (table 3). This was neither due to interference from uroporphyrinogen nor to uroporphyrin [17]. Inhibition was significantly less if uroporphyrin III replaced uroporphyrinogen III in the oxidation system.

4. DISCUSSION

It appears that hydroxyl radicals (or related iron-oxygen species) can oxidise uroporphyrinogen to uroporphyrin. Perhaps more importantly, side products of greater polarity are formed that cannot regain the porphyrin structure. It is interesting, however, that although superoxide dismutase decreased the amount of side products formed it enhanced uroporphyrin formation which may imply that 'softer' oxidising species were produced capable of abstracting hydrogen from uroporphyrinogen but incapable of inserting oxygen functions. Variations in such a balance of oxidising species may have parallels in isolated hepatocytes and in vivo. Not surprisingly, hydroxymethylbilane (the biosynthetic precursor of uroporphyrinogens [18]) is also attacked by hydroxyl radicals.

The hypothesis that some modified porphyrinogens are responsible for uroporphyrinogen decarboxylase inhibition in vivo is supported by the data in table 3. Although inhibitions observed were moderate, it is worth noting that the total products in the assays were in the nanomolar range and in vivo oxidations might well be considerably more selective. Studies are in progress to examine polar products of uroporphyrinogen oxidation and to explore any similarities with the inhibitor of uroporphyrinogen decarboxylase detected in mouse liver after treatment of animals with various aromatic drugs and environmental chemicals [5,19,20].

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