Inhibition of collagen hydroxylation by 2,7,8-trihydroxyanthraquinone in embryonic-chick tendon cells

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Prolyl 4-hydroxylase (EC 1.14.11.2) is an essential enzyme in the post-translational modification of collagen. Inhibitors of this enzyme are of potential interest for the treatment of diseases involving excessive deposition of collagen. 2,7,8-Trihydroxyanthraquinone (THA) is an effective inhibitor of prolyl 4-hydroxylase by virtue of its ability to compete with the co-substrate 2-oxoglutarate ($K_i = 40.3 \mu\text{M}$). Using a simple and reproducible assay for collagen hydroxylation, we show that THA inhibits the hydroxylation of collagen in embryonic-chick tendon cells in short-term culture, with an IC_{50} value (concn. giving 50% inhibition) of $32 \mu\text{M}$. In comparison, the ethyl ester of 3,4-dihydroxybenzoic acid has an IC_{50} value of 0.1 mM against collagen hydroxylation by chick tendon cells, whereas its K_i versus isolated prolyl 4-hydroxylase is $49 \mu\text{M}$.

INTRODUCTION

The hydroxylation of certain prolyl residues of collagen pro- α -chains is essential for the formation of the characteristic triple helix of the mature collagen molecule. Since secretion of collagen into the extracellular matrix only occurs when the molecule is in the triple-helical form (Uitto & Prockop, 1974), the principal enzyme involved in collagen hydroxylation, prolyl 4-hydroxylase (EC 1.14.11.2), is seen as a potential target for drugs that could be used to treat a range of serious diseases characterized by excessive deposition of collagen. These include fibrosis of the lungs and liver, scleroderma and the proliferative pannus of rheumatoid arthritis. Although several inhibitors of prolyl 4-hydroxylase have been described, their usefulness against intact cells has been limited by several factors, including lack of specificity, e.g. the antitumour antibiotic P-1894B (Ishimaru *et al.*, 1982) and 2,2'-bipyridyl (Tschank *et al.*, 1987a), and difficulty in crossing the cell membrane, as with several pyridinedicarboxylic acids (Tschank *et al.*, 1987a) and possibly the ethyl ester of 3,4-dihydroxybenzoic acid (Sasaki *et al.*, 1987). A previous report from our laboratory (Cunliffe & Franklin, 1986) described the inhibition of chick prolyl 4-hydroxylase by a series of hydroxyanthraquinones having at least two hydroxy groups *ortho* to each other. In the present paper we show that one of these compounds, 2,7,8-trihydroxyanthraquinone (THA), is a potent non-toxic inhibitor of collagen hydroxylation in embryonic-chick tendon cells *in vitro*.

MATERIALS AND METHODS

Materials

L-[U- ^{14}C]Proline (262 mCi/mmol) and L-[4- ^3H (n)]-proline (21 Ci/mmol, 63% of ^3H in the 4-*trans* position) were both obtained from New England Nuclear Research Products, Boston, MA, U.S.A. Collagenase (type 1A), ascorbic acid (sodium salt) and 2,2'-bipyridyl were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Trypsin (cat. no. 043-5090) was from Gibco, Paisley,

Scotland, U.K., and the ethyl ester of 3,4-dihydroxybenzoic acid (EDHB) was from Aldrich Chemical Co., Gillingham, Dorset, U.K. THA was synthesized in our laboratories by Mr. R. I. Dowell and Mrs. E. M. Hadley.

Isolation of embryonic-chick tendon cells

Cells were isolated from the leg tendons of 17-day chick embryos by a modification of a previous procedure (Dehm & Prockop, 1971). The tendons were incubated in air, rather than in O_2/CO_2 (19:1), with a mixture of trypsin and collagenase in Eagle's minimal essential medium, pH 7.4, containing 20 mM-Hepes and agitated with a plastic pipette every 10 min for a total of 40 min to assist the dispersion of the cells. The enzyme digestion was repeated for a second period of 40 min on the undigested tendon material remaining at the end of the first period. Proteolysis was arrested by the addition of foetal-calf serum (final concn. 10%, v/v). The cell suspension was filtered twice through lens tissue, the tissue being rinsed with Eagle's medium each time to wash through adherent cells.

Assay of collagen hydroxylation in chick tendon cells

Cells, derived as above, were sedimented at 600 *g* for 5 min and resuspended in Krebs' modified medium (Dehm & Prockop, 1971) containing 10% foetal-calf serum (KF medium). Cell viability, determined by the exclusion of Trypan Blue, was considered acceptable at > 95%. Viable cells were diluted to $10^7/\text{ml}$ in KF medium and dispensed (1 ml) into small plastic bottles (7 ml; Sterilin cat. no. 129B). The incubation mixture was completed by addition of ascorbate (25 mM), $1 \mu\text{Ci}$ of L-[^{14}C]proline and $4 \mu\text{Ci}$ of L-[^3H]proline in a total volume of 2.5 ml of KF medium. The bottles were capped and incubated at 37 °C on a blood-mixer roller. In some experiments cell viabilities were determined by the dye-exclusion method at the end of the incubation period. Metabolic activity was arrested after 3 h by chilling in ice and adding ice-cold EDTA (final concn. 1 mM). The cell suspensions were transferred to centrifuge

Abbreviations used: THA, 2,7,8-trihydroxyanthraquinone; IC_{50} , concentration giving 50% inhibition; EDHB, ethyl ester of 3,4-dihydroxybenzoic acid.

tubes together with two washes (1 ml) of the incubation bottles with ice-cold EDTA (1 mM). Ice-cold trichloroacetic acid was added to a final concentration of 10% (w/v), and the tubes were left on ice for 1 h. The precipitated proteins were sedimented by centrifugation and resuspended twice in ice-cold trichloroacetic acid (5%).

Incorporation of radiolabel into collagen was determined by a modification of the collagenase procedure of Peterkofsky & Diegelmann (1971). Each protein precipitate was dissolved in 0.2 M-NaOH (1 ml). Duplicate samples (400 μ l) were transferred to centrifuge tubes together with the following: 1 M-Hepes (260 μ l), 0.15 M-HCl (260 μ l), 62.5 mM-N-ethylmaleimide (80 μ l), 25 mM-CaCl₂ (40 μ l), 0.26 M-Hepes (960 μ l). To one tube of each duplicate pair was added 160 units (as defined for Sigma product no. C-0773) of collagenase (10 μ l). The tubes were sealed and incubated at 37 °C for 90 min. Digestion was stopped with ice-cold trichloroacetic acid (final concn. 10%) by using bovine serum albumin (final concn. 20 mg/ml) as a co-precipitant. The precipitated proteins were sedimented (27000 g for 5 min) and the ratio of ³H/¹⁴C was determined by liquid-scintillation counting. Control studies with protein from cells labelled with [¹⁴C]tryptophan indicated that collagenase digestion did not cause any solubilization of radioactivity from non-collagenous proteins (T. J. Franklin & M. Hitchen, unpublished work). The determination of the extent of hydroxylation of prolyl residues at the 4-position in collagenase-solubilized material was based on the elimination of ³H from incorporated prolyl residues (Chojkier *et al.*, 1980). Since hydroxylation only occurs at the 4-*trans* position, it was important to take account of the proportion of ³H in this position in the added labelled proline (63%). Owing to hydroxylation, the ratio of ³H to ¹⁴C in collagen is normally smaller than that in non-collagenous proteins and in collagen when hydroxylation is suppressed by inhibitors of hydroxylation such as 2,2'-bipyridyl. In the comparison of inhibitors of hydroxylation, the extent of incorporation of [¹⁴C]proline provides a useful check for general toxic effects of compounds on collagen synthesis. The percentage hydroxylation of prolyl residues in the 4-*trans* position is given by the expression:

$$\left[\frac{1 - \frac{{}^3\text{H}/{}^{14}\text{C} \text{ ratio in supernatant of collagenase digest (control cells)}}{{}^3\text{H}/{}^{14}\text{C} \text{ ratio in supernatant of collagenase digest (2,2'-bipyridyl-treated cells)}}}{\text{fraction of } {}^3\text{H} \text{ in 4-} \textit{trans} \text{ position of proline}} \right] \times 100$$

2,2'-Bipyridyl was used at a concentration (1 mM) sufficient to suppress hydroxylation completely.

RESULTS

Reproducibility of collagen hydroxylation

In a series of 21 experiments using separate batches of embryonically derived cells, the percentage hydroxylation of collagenase-solubilized peptides was 38.6% $\pm$ 0.33 (S.E.M.). Assuming that the collagen produced by embryonic tendon cells is mainly Type 1, this value appears rather low, since the percentage hydroxylation of proline in this collagen is about 44% (Kivirikko & Myllyla, 1980). However, the latter is the value for mature extracellular collagen, whereas the collagenase-soluble

Fig. 1. Inhibition of collagen hydroxylation in embryonic-chick tendon cells by THA

Cells were incubated for 3 h with [¹⁴C]- and [³H]-proline and the indicated concentrations of THA. The extent of hydroxylation of collagenase-soluble peptides prepared from cells and medium combined was determined as described in the text. Values are means $\pm$ S.E.M. of the results from three experiments, in which there were eight replicate incubations for each point on the curve.

material evaluated in the present study is a mixture of collagen secreted into the medium and intracellular collagen. A significant proportion of the latter is likely to be incompletely hydroxylated. The reproducibility of the percentage hydroxylation in a long series of experiments encouraged the view that the procedure for measuring hydroxylation described in the present paper could be used to compare the effects of inhibitors of prolyl 4-hydroxylase on the intracellular hydroxylation of collagen.

Inhibition of collagen hydroxylation by THA

Three separate experiments, with different batches of cells, were performed to establish the effects of THA. The results, summarized in Fig. 1, indicate a sigmoidal dose-response relationship similar to that seen with THA on the isolated enzyme (Cunliffe & Franklin, 1986). A 50% inhibition of collagen hydroxylation in the tendon cells was achieved at a mean concentration of 32 μ M (IC₅₀). The effect of THA on the incorporation of [¹⁴C]proline into total collagen, i.e. collagenase-soluble material in cells and medium combined, at a concentration which completely suppressed collagen hydroxylation was considerably smaller than that produced by the concentration of 2,2'-bipyridyl typically used to

Table 1. Effects of inhibitors of prolyl 4-hydroxylase on collagen hydroxylation, [^{14}C]proline incorporation into collagen and cell viability in suspensions of embryonic-chick tendon cells

Values are means $\pm$ S.E.M. of replicate (n) incubations (3 h) of cells drawn from a single pooled preparation. [^{14}C]Proline incorporation into collagenase-soluble material, hydroxylation of collagen and cell viability at the end of the incubation were determined as described in the text.

Addition	[^{14}C]Proline incorporation into collagen (d.p.m./ 10^6 cells)	Hydroxylation of collagen (%)	Ratio of $^3\text{H}/^{14}\text{C}$ in collagen
None ($n = 6$)	21 854 $\pm$ 1059	38.4	3.26 $\pm$ 0.09
2,2'-Bipyridyl (1 mM) ($n = 4$)	4032 $\pm$ 138	0	4.26 $\pm$ 0.10
THA (0.078 mM) ($n = 6$)	17 597 $\pm$ 1381	0	4.25 $\pm$ 0.06
EDHB (0.219 mM) ($n = 4$)	16 523 $\pm$ 536	0	4.25 $\pm$ 0.1

suppress hydroxylation (Table 1); cf. Chojkier *et al.* (1980) and Dehm & Prockop (1971). THA may also have had a smaller effect on cell viability over 3 h (95 % viability after 3 h incubation) than 2,2'-bipyridyl (90 % viability). In all further investigations of inhibitors of collagen hydroxylation in tendon cells, therefore, we substituted THA for 2,2'-bipyridyl as an agent to suppress hydroxylation. The ratio of $^3\text{H}/^{14}\text{C}$ in collagenase-soluble peptides from cells incubated with 78 μM -THA (Table 1) then replaced that obtained with 2,2'-bipyridyl in the expression to calculate percentage hydroxylation.

Inhibition of collagen hydroxylation in tendon cells by EDHB

EDHB has been reported to be considerably more effective as an inhibitor of collagen hydroxylation in cultures of human keloid fibroblasts than its less hydrophilic parent compound 3,4-dihydroxybenzoic acid (Sasaki *et al.* 1987). Although a complete dose-response curve was not reported, EDHB inhibited hydroxylation by 80 % at 0.4 mM and 39 % at 0.2 mM. In a series of 20 experiments with different batches of cells, we observed a mean inhibition of collagen hydroxylation by EDHB in chick tendon cells of 43.1 % $\pm$ 2.9 (S.E.M.) at 0.109 mM (20 $\mu\text{g}/\text{ml}$). A full dose-response study gave an IC_{50} of 0.1 mM against collagen hydroxylation in chick tendon cells (Fig. 2). This compound therefore appears to have comparable activity against human keloid cells and embryonic-chick tendon cells. The effect of EDHB on the incorporation of [^{14}C]proline into collagen at a concentration which completely suppressed hydroxylation in chick tendon cells was comparable with that of THA (Table 1). Using the method described by Cunliffe *et al.* (1986) for the assay of isolated prolyl 4-hydroxylase, we found K_i values for EDHB and the parent free acid against purified chick prolyl 4-hydroxylase of 49 μM and 42 μM respectively (results not shown). Thus 3,4-dihydroxybenzoic acid and its ethyl ester were approximately equiactive against prolyl hydroxylase, although

Fig. 2. Inhibition of collagen hydroxylation in embryonic-chick tendon cells by EDHB

Cells were incubated for 3 h with [^{14}C]- and [^3H]-proline and the indicated concentrations of EDHB. The extent of hydroxylation of collagenase-soluble peptides prepared from cells and medium combined was determined as described in the text. Values are means $\pm$ S.E.M. of the results from three experiments, in which there were eight replicate incubations for each point on the curve.

the possibility of partial hydrolysis of the ester to the free acid during incubation with the enzyme cannot be ruled out.

DISCUSSION

Conventional procedures for measuring the hydroxylation of prolyl residues in collagen pro- α chains usually rely on monitoring the incorporation of radiolabelled proline into hydroxyproline. This requires the separation of hydroxyproline from proline in protein hydrolysates by chromatography. Chojkier *et al.* (1980) showed that proline hydroxylation in guinea-pig calvaria *in vitro* could be determined by measuring the simultaneous incorporation of [^{14}C]proline and [^3H]proline into the collagenase-soluble fraction. The ratio of radiolabels in collagen obtained in the presence of 2,2'-bipyridyl was used as an indicator of zero hydroxylation. We have found that an adaptation of this method for suspensions of embryonic tendon cells is a convenient and reliable way of determining the extent of hydroxylation of prolyl residues in the 4-position in these cells *in vitro*.

Using this technique, we found that THA is among the most potent inhibitors of intracellular collagen hydroxylation so far reported, with an IC_{50} of 32 μM . The K_i for this compound against purified chick prolyl 4-hydroxylase is 40.3 μM (Cunliffe & Franklin, 1986). It should be borne in mind that the K_i for THA was determined in the presence of 50 μM - Fe^{2+} , the concentration required for optimal enzyme activity. The inhibitory potency of THA against prolyl 4-hydroxylase increased in the absence of Fe^{2+} , presumably a reflection of the chelating affinity of THA for Fe^{2+} in solution (Cunliffe & Franklin, 1986). Since the prevailing concentration of free Fe^{2+} at the

intracellular site of prolyl hydroxylase is likely to be much lower than $50\text{ }\mu\text{M}$, the 'real' K_i of THA will be considerably less than $40.3\text{ }\mu\text{M}$. Furthermore, because no value is available for the concentration of 2-oxoglutarate (the co-substrate antagonized by THA) at the intracellular site of prolyl hydroxylase, we cannot directly relate the IC_{50} value against collagen hydroxylation to the observed K_i value against the isolated enzyme. It is possible that THA may diffuse relatively freely across the cellular permeability barriers to the intracellular site of the enzyme, although direct evidence for this is lacking. Unlike 2,2'-bipyridyl, THA had only a minor effect on the incorporation of [^{14}C]proline into collagen at a concentration that completely suppressed the 4-hydroxylation of prolyl residues.

3,4-Dihydroxybenzoic acid has a K_i against chick prolyl hydroxylase of approx. $46\text{ }\mu\text{M}$ (Majamaa *et al.*, 1986; the present work), but the compound had little or no effect on collagen hydroxylation in human keloid fibroblasts *in vitro* at concentrations between 0.2 and 0.4 mM (Sasaki *et al.*, 1987) or in chick tendon cells (T. J. Franklin & M. Hitchen, unpublished work). Sasaki *et al.* (1987) therefore synthesized the ethyl ester of 3,4-dihydroxybenzoic acid (EDHB) in an attempt to produce a hydrophobic derivative which would diffuse across the permeability barriers to the intracellular site of the enzyme. However, even the ester required a concentration of 0.2 mM to inhibit hydroxyproline synthesis by approx. 40% (Sasaki *et al.*, 1987). The results in the present paper show that EDHB has similarly modest activity against collagen hydroxylation in embryonic-chick tendon cells, with an IC_{50} value of 0.1 mM. The discrepancy between the inhibitory potency of EDHB against prolyl hydroxylase and against intracellular hydroxylation in both human and chick cells may have been due to slow penetration into the cells, despite greater hydrophobicity compared with the free acid. However, work by Tschank *et al.* (1987b) indicates that the mechanism of inhibition of prolyl hydroxylase by 3,4-dihydroxybenzoic acid in preparations of endoplasmic reticulum may be different from that which inhibits the purified enzyme. They speculate that intracellular conversion of the catechol into a highly reactive molecule in the presence of metal ions and oxygen might lead to the inactivation of postulated specific uptake processes for 2-oxoglutarate and ascorbate into the vesicles of the endoplasmic reticulum. Unlike 3,4-dihydroxybenzoic acid, THA is not a competitive inhibitor of ascorbate action, but exhibits positively co-operative competition with 2-oxoglutarate (Cunliffe & Franklin, 1986). The sigmoidal shape of the dose-response for THA against collagen hydroxylation in tendon cells points to a possibly similar interaction with the enzyme at its intracellular site. The minimal effects of THA on collagen synthesis, as indicated by the incorporation of [^{14}C]proline into collagenase-soluble material and cell viability, suggest that the intracellular formation of toxic derivatives of THA may be insignificant. There is no reason at present, therefore, to believe that the mechanism of inhibition of intracellular collagen hydroxylation by THA differs from that of the isolated enzyme, although this possibility cannot be ruled out.

Metal ions known to inhibit prolyl hydroxylase have also been used to inhibit collagen hydroxylation in cultured cells and tissue explants. Antinnen *et al.* (1980) found that $10\text{ }\mu\text{M}$ - Zn^{2+} inhibited prolyl hydroxylation in chick tendon cells by approx. 35%. Co^{2+} achieved a similar effect at 0.1 mM. Rapaka *et al.* (1976) showed that 0.6 mM - Pd^{2+} inhibited hydroxyproline synthesis in embryonic-chick cartilage explants by 90% or more. However, in contrast with THA, concentrations of Zn^{2+} and Pd^{2+} which depressed collagen hydroxylation also inhibited collagen synthesis.

In conclusion, therefore, we have demonstrated that THA inhibits the prolyl 4-hydroxylation of collagen in tendon cells *in vitro* at concentrations which approximate to those that inhibit isolated prolyl 4-hydroxylase and which cause minimal toxicity to the cells. The compound would be expected to prevent the secretion of collagen into the extracellular matrix, since this process depends on the hydroxylation of specific proline residues in procollagen. Recent results have confirmed that, when hydroxylation is inhibited by THA, embryonic-chick tendon cells fail to release collagen into the medium (T. J. Franklin & S. M. Hitchen, unpublished work). Although the compound lacks the potency required of a drug, our findings should encourage the search for more potent inhibitors which could be used to treat the serious diseases characterized by the hypersecretion of collagen into the extracellular matrix.

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