INHIBITION OF PROLINE HYDROXYLATION DOES NOT INHIBIT SECRETION OF TROPOELASTIN BY CHICK AORTA CELLS

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1. Introduction

In vertebrates, the protein elastin is a vital component of major blood vessels, imparting to them their elastic, rubber-like quality. Purified elastin is very inert and insoluble due to extensive crosslinking of the polypeptide chains by desmosines and other cross-linkages [1,2] derived from the oxidation of lysine residues by a copper-requiring enzyme [3–5]. Sandberg, et al. [6] prepared a soluble protein called tropoelastin, from the aortae of copper-deficient pigs and this protein consisted of a single polypeptide chain with an estimated mol. wt. of about 70 000 and an amino acid composition which closely resembled that of insoluble elastin except for the absence of the desmosines. A significant portion of the tropoelastin molecule has been sequenced [7] and a model for cross-linked elastin has been proposed based on this data [8]. A similar protein has been isolated from the aortae of copper-deficient or lathyritic chicks [9,10].

We have previously reported that isolated embryonic chick aortae synthesize a soluble protein whose labeling pattern with radioactive amino acids was suggestive of that of tropoelastin and which migrated close to serum albumin (68 000 daltons) during gel electrophoresis in sodium dodecyl sulfate [11]. During pulse-chase experiments this soluble protein was incorporated into an insoluble residue. Smith and Carnes [12] have demonstrated that when aortae from normal newborn pigs were incubated in vitro, they synthesize a labeled protein which manifests many of the properties of added carrier tropoelastin. Cells isolated from the media of pig aortae can synthesize elastin after they have been in long term culture [13].

Recently we have shown that cells isolated from embryonic chick aortae can synthesize and secrete procollagen and tropoelastin into the surrounding medium when incubated in suspension culture under the appropriate conditions [14]. In the present communication we report that incubation of these cells with concentrations of α,α'-dipyridyl which totally inhibit proline hydroxylation markedly decreases the rate of procollagen secretion but does not affect the rate of tropoelastin secretion.

2. Materials and methods

Cells were isolated from 17-day chick embryo aortae as previously described [14]. Briefly, a section of the thoracic aorta extending from the root to a point just proximal to the entrance of the ductus arteriosus was dissected with sterile precautions. The innominate arteries to the point of their bifurcation into the subclavian and carotids were included. Loose connective tissue was stripped from the aortae which were cut along their long axis and then minced. The small fragments were resuspended in Eagle's minimal media containing 2% fetal calf serum and 1 mg/ml purified collagenase (Worthington) and 2.5 mg/ml trypsin (Grand Island Biologicals). 1 ml of media equilibrated with 5% CO_2, 95% air was used per 12–15 aortae and the aortae were shaken in a water bath. At the end of 1 h of digestion at 37°C the fragments were essentially completely solubilized. The suspension

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was passed through lens paper and the filtrate centrifuged at 1200 *g* for 5 min. The cells were washed three times by resuspension and centrifugation in Krebs-Ringer containing 2% calf serum. Approx. 3 million cells were obtained per aorta.

The cells were incubated at a concentration of 2 million cells per ml in Krebs-Ringer containing 2% fetal calf serum, 25 μg/ml of ascorbate and 200 μg/ml pancreatic trypsin inhibitor (Worthington) and [^{14}C]proline (200 μCi/μmole, New England Nuclear) with or without a variable concentration of α,α′-dipyridyl (Eastman Kodak) for variable periods of time. The trypsin inhibitor was necessary to inactivate residual trypsin left from the cell isolation procedure. Details of particular experiments are given in the table and figures. The [^{14}C]hydroxyproline content of some samples was measured by the method of Juva and Prockop [15].

Disc gel electrophoresis in sodium dodecyl sulfate was performed as previously described [11] using 5% acrylamide gels and one-half the standard amount of cross-linker. The gels were immediately chilled and cut into 1.5 mm fractions in order to minimize losses, and the fractions were solubilized with 30% H_2O_2 and counted in a scintillation counter. Duplicate gels were run containing radioactive samples, purified α and β chains of rat tail collagen and bovine serum albumin. These gels were stained with Coomassie blue and the radioactive peaks were identical in the stained and unstained gels when corrected for gel swelling during destaining.

3. Results and discussion

When the cells were incubated with concentrations of α,α′-dipyridyl varying between 10^{-5} M and 2×10^{-4} M, the hydroxylation of proline was progressively inhibited, so that at 10^{-4} M less than 0.5% of the proline residues were hydroxylated compared to a value of 12.0% in the control (table 1). Correlated with this inhibition of hydroxylation there was a decrease in the fraction of the labeled proteins which were secreted into the media. In the control after 90 min of incubation, the ratio of secreted to intracellular proteins was 0.46 while in the samples in which hydroxylation was essentially completely inhibited this ratio declined to 0.22–0.25.

In order to study the kinetics of secretion of the labeled proteins during inhibition of proline hydroxylation and to characterize these proteins in comparison to control conditions, cells were incubated with

Table 1
Effect of inhibition of proline hydroxylation on the secretion of [^{14}C]proline-labeled proteins

α,α′-Dipyridyl Concentration (M)	Degree of hydroxylation[a] (%)	Secreted (S) (cpm × 10^{-3})	Intracellular (I) (cpm × 10^{-3})	$\frac{(S)}{(I)}$
0	12.0	105.3	229.7	0.46
10^{-5}	11.0	99.5	225.5	0.44
5×10^{-5}	1.1	40.3	166.4	0.24
10^{-4}	0.2	36.5	164.2	0.22
2×10^{-4}	0.2	37.0	146.9	0.25

Chick arota cells (2×10^6/ml) were incubated as described in Materials and methods with 3 μCi/ml [^{14}C]proline and the concentrations of α,α′-dipyridyl listed in the table. After 90 min of incubation, 1 ml aliquots of the cell suspensions were taken and centrifuged. The cells were resuspended in 1 ml of 0.01 M phosphate buffer, pH 7.4, and sodium dodecyl sulfate and mercaptoethanol were added to final concentrations of 1% to the cells and media. The samples were then placed in a boiling water bath for 2 min and dialyzed against 0.1% sodium dodecyl sulfate and mercaptoethanol. After dialysis, aliquots were counted in a scintillation counter.

[a]Values are 100 times [^{14}C]hydroxyproline per total ^{14}C in the whole system (secreted plus intracellular).

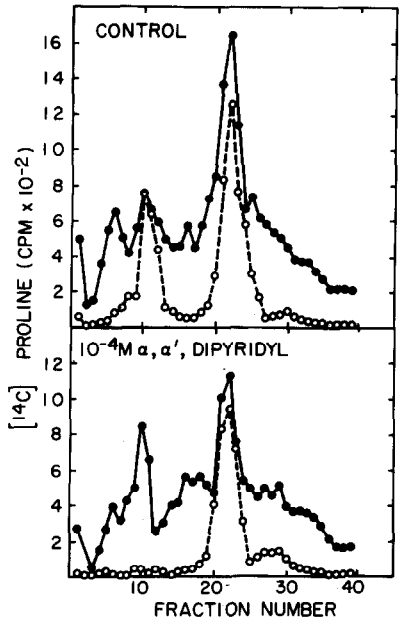

Fig.1. Polyacrylamide disc gel electrophoresis of intracellular and extracellular labeled proteins synthesized by aorta cells in the presence or absence of 10^{-4} M α,α'-dipyridyl. Aliquots of cells which had been incubated for 120 min were treated as described in table 1. Electrophoresis and counting of the gels were then carried out as described in Materials and methods. Marker β chains of rat tail collagen were located at fraction 8, α chains of rat tail collagen at fraction 13, and bovine serum albumin at fraction 22–23. Intracellular (● - ● - ●); extracellular (○ - ○ - ○).

10^{-4} M α,α'-dipyridyl and [^{14}C]proline for 2 h. This concentration was chosen since it was the minimal α,α'-dipyridyl concentration which consistently inhibited hydroxylation completely. At 30, 60 and 120 min, 1 ml samples of the cell suspensions were taken and the labeled intracellular and secreted proteins were subjected to electrophoresis on polyacrylamide gels in sodium dodecyl sulfate. Fig.1 illustrates the patterns obtained from the 120 min incubation samples. In the control sample two major peaks were observed in the secreted proteins. The larger 125 000 mol. wt. component has been shown to be a form of procollagen while the 70 000 mol. wt. component is a form of tropoelastin [11,14]. Peaks in the same positions of the gel were also observed in the control intracellular sample, although as one would expect, the distribution of radioactivity was more heterogeneous. In the experimental sample of the secreted proteins, the quantity of radioactivity found in the procollagen position was greatly diminished while the quantity in the tropoelastin position was only slightly decreased. The intracellular experimental proteins contained distinct peaks in the procollagen and tropoelastin positions. The amount of labeled procollagen and tropoelastin in each of the samples was estimated by summing the radioactivity in the appropriate peak fractions of the gels. The results of these determinations are illustrated in fig.2 for procollagen and fig.3 for tropoelastin. These results demonstrate that in the control after a lag of about

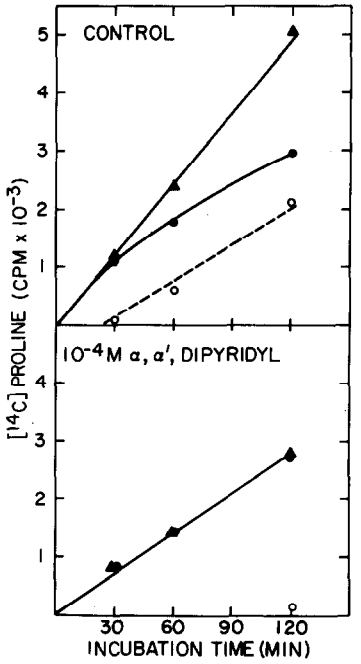

Fig.2. Synthesis and secretion of procollagen by aorta cells incubated with or without 10^{-4} α,α'-dipyridyl. Aorta cells were incubated with 3 μCi/ml of [^{14}C]proline and at the times indicated 1 ml aliquots of the cell suspensions were centrifuged and the labeled intracellular and extracellular proteins prepared for electrophoresis as described in table 1 and subjected to polyacrylamide disc gel electrophoresis as in fig.1. By summation of the counts in the procollagen region (usually fractions 9–12), an estimate of the radioactivity in intracellular and extracellular procollagen was made. These measurements were then plotted as a function of incubation time. Intracellular procollagen (● - ● - ●); extracellular procollagen (○ - ○ - ○); intracellular and extracellular (▲-▲-▲).

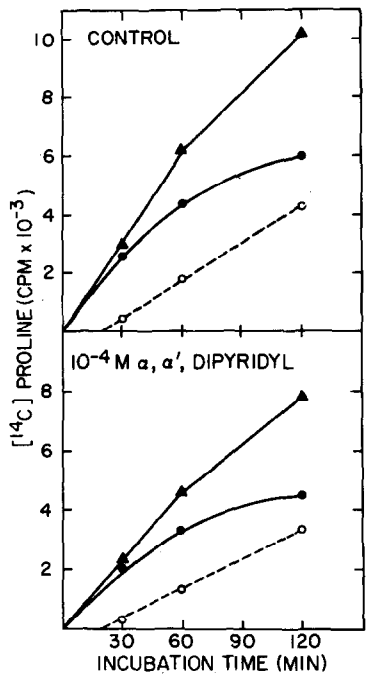

Fig.3. Synthesis and secretion of tropoelastin by aorta cells incubated with or without 10^{-4} M α,α′-dipyridyl. Aorta cells were incubated with [^{14}C]proline and aliquots of the intracellular and secreted proteins were subjected to polyacrylamide gel electrophoresis as described in fig.1. By summation of the counts in the tropoelastin region (usually fractions 20–24), an estimate of the radioactivity in intracellular and extracellular tropoelastin was made. These measurements were then plotted as a function of incubation time. Intracellular tropoelastin (● - ● - ●); extracellular tropoelastin (○ - ○ - ○); intracellular and extracellular (▲-▲-▲).

23 min, procollagen is secreted into the medium at a linear rate. However, in the incubation with α,α′-dipyridyl no secreted procollagen was detected in the 30 min and 60 min samples. In the 120 min sample the amount of secreted procollagen was less than 5% of that found in the control. The amount of procollagen found intracellularly was slightly less at all time points in the experimental samples in comparison to the control samples. The results of the tropoelastin determinations were quite different. Although the quantity of tropoelastin secreted in the presence of 10^{-4} M α,α′-dipyridyl at each time point was about 80% of the control, the time of first appearance of tropoelastin in the media was identical within experimental error, to that of the control. By projection onto the time axis of the straight line obtained from the secreted values, we estimate that there was a lag of about 19 min from the time of initiation of labeling until the first appearance of labeled tropoelastin in the media. Similar results were found in an identical experiment carried out with 2×10^{-4} M α,α′-dipyridyl (data not shown).

The finding that inhibition of hydroxylation markedly delayed the secretion of procollagen is consistent with previous findings using fibroblasts isolated from embryonic chick tendons as well as other fibroblasts [16–18].

Since unhydroxylated chick procollagen has been shown to be in a non-triple helical conformation at 37°C in contrast to the hydroxylated triple helical control state [19], the intracellular retention of the unhydroxylated molecules suggested that the proper conformation of procollagen is important for normal secretion. Alternatively, prolyl and/or lysyl hydroxylase under inhibitory conditions of hydroxylation may form a tight enzyme–substrate complex which delays secretion. The results using the proline analog, *cis* -hydroxyproline, in which incorporation of the analog destabilizes the triple helix but in which the intracellularly retained molecules do not bind tightly to prolyl hydroxylase support the conformation theory [20,21]. However, whatever proves to be the correct explanation for the findings with procollagen, the results with tropoelastin secretion were markedly different. Although the total incorporation of label into tropoelastin was slightly decreased in the presence of 10^{-4} M α,α′-dipyridyl, the delay in the first appearance of tropoelastin in the media was identical to that of the control. Chick aorta tropoelastin has been reported to contain about 10 residues hydroxyproline per thousand residues while insoluble elastin contains about 20 residues hydroxyproline per thousand and chick collagen 95 residues hydroxyproline per thousand [9,10]. We report elsewhere [22] that an enzyme either identical or very similar to collagen prolyl hydroxylase can also hydroxylate tropoelastin. However, we do not as yet have any information about the binding of tropoelastin to prolyl hydroxylase in the absence of hydroxylation nor do we know whether the absence of hydroxyproline in the tropoelastin alters the conformation of the molecule significantly.

Although procollagen secretion was significantly inhibited in the absence of hydroxylation, no greater amounts of procollagen were found intracellularly than in the control. The situation is complicated by the fact that α,α'-dipyridyl appeared to produce a moderate inhibition of incorporation of [^{14}C]proline into a protein such as tropoelastin which is secreted. Also, we do not know whether a single cell is simultaneously synthesizing procollagen and tropoelastin. However, specific feedback mechanisms may exist which coordinate the synthesis of procollagen with its secretion so that when secretion is inhibited the rate of synthesis is decreased.

Acknowledgements

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