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Biochemistry

Regulation of collagen synthesis by ascorbic acid

(prolyl hydroxylation/lysyl hydroxylation/human skin fibroblasts)

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ABSTRACT **After prolonged exposure to ascorbate, collagen synthesis in cultured human skin fibroblasts increased approximately 8-fold with no significant change in synthesis of noncollagen protein. This effect of ascorbate appears to be unrelated to its cofactor function in collagen hydroxylation. The collagenous protein secreted in the absence of added ascorbate was normal in hydroxylysine but was mildly deficient in hydroxyproline. In parallel experiments, lysine hydroxylase (peptidyllysine, 2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) activity increased 3-fold in response to ascorbate administration whereas proline hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) activity decreased considerably. These results suggest that collagen polypeptide synthesis, posttranslational hydroxylations, and activities of the two hydroxylases are independently regulated by ascorbate.**

Ascorbic acid is essential for normal collagen formation (1–3) by virtue of the fact that it is a required component in the synthesis of hydroxyproline and hydroxylysine in collagen (4). Hydroxyproline serves to stabilize the collagen triple helix (5, 6); its absence results in structurally unstable collagen (7, 8) which is not secreted from cells at a normal rate (9). Hydroxylysine is necessary for formation of the intermolecular crosslinks in collagen (10). In addition, specific carbohydrate residues are linked glycosidically to collagen through hydroxylysine, a process that may be important in the regulation of crosslink formation (11).

It is generally believed that ascorbate modulates collagen production through its effect on prolyl hydroxylation (12). There have been indications, however, that ascorbate may have an additional role in collagen biosynthesis (13–16). Notable are the early studies by Jeffrey and Martin (13) who observed a substantial increase in the size of chicken long bones cultured in the presence of ascorbate, concomitant with an increase in the incorporation of proline into peptidyl hydroxyproline.

In this study we have examined the long-term effect of ascorbate on collagen production by cultured human skin fibroblasts. The influence of ascorbate on prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) and lysyl hydroxylase (peptidyllysine, 2 oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) levels was also examined simultaneously to understand better the interrelationship of collagen synthesis and posttranslational hydroxylation. The data indicate that ascorbate increases collagen synthesis by acting at a level other than hydroxylation.

MATERIALS AND METHODS

Human skin fibroblasts from a normal 3-day-old boy (GM 970) were obtained from the Institute for Medical Research (Camden, NJ) and grown to confluent density in Dulbecco's modified Eagle's medium buffered with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with 20% fetal calf serum (GIBCO) which had been inactivated for 30 min at 56°C. Cultures were growth arrested for 90 hr in the medium as above but containing 0.5% dialyzed fetal calf serum. Medium was changed each 48 hr. L-Ascorbic acid or D-isoascorbic acid solution was prepared fresh daily and added for 0, 24, or 96 hr prior to harvest. Washed cells were incubated for the final 6 hr in lysine-free Dulbecco's modified Eagle's medium buffered with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with glucose at 3.5 mg/ml, 0.5% dialyzed fetal calf serum, 0.2 mM β-aminopropionitrile fumarate, 30 μCi of L-[2,3-^{3}H]proline (New England Nuclear; 1 Ci = 3.7×10^{10} becquerels), and 4 μCi of L-[^{14}C]lysine (New England Nuclear). After incubation, protease inhibitors were added to the medium in the following concentrations: *N*-ethylmaleimide, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; EDTA, 1 mM. The cell layer was harvested with 0.05% trypsin/0.5 mM EDTA and an aliquot was assayed in the Coulter Counter. Cultures were studied in triplicate.

For enzyme studies, parallel cultures were established as described above except the labeling medium was replaced by Dulbecco's modified Eagle's medium buffered with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with 0.5% dialyzed fetal calf serum. Medium was changed daily.

Measurement of Collagen Synthesis. Radioactivity incorporated into collagen was determined after digestion with highly purified bacterial collagenase (form III, Advance Biofactures) (17, 18). For amino acid analysis, aliquots of collagenase-digested medium, after precipitation of nondigested protein by 25% trichloroacetic acid/1.25% tannic acid, were hydrolyzed in 6 M HCl for 20 hr at 108°C.

Isolation of Procollagen I. Procollagen I was isolated by DEAE-cellulose chromatography (17, 19). For amino acid analysis, pooled samples were hydrolyzed in 6 M HCl for 20 hr at 108°C.

Amino Acid Analysis. Amino acids were separated on a Beckman model 116 amino acid analyzer. Radioactivities in proline, hydroxyproline, lysine, and hydroxylysine were determined by liquid scintillation counting in Aquasol-2 (New England Nuclear).

Prolyl and Lysyl Hydroxylase Assays. Prolyl and lysyl hydroxylase activities in cell extracts were determined essentially as described (20).

RESULTS

Total collagen production was stimulated approximately 8-fold by L-ascorbate as well as D-isoascorbate (Fig. 1). Cell pellet collagen represented a greater fraction of total collagen in as-

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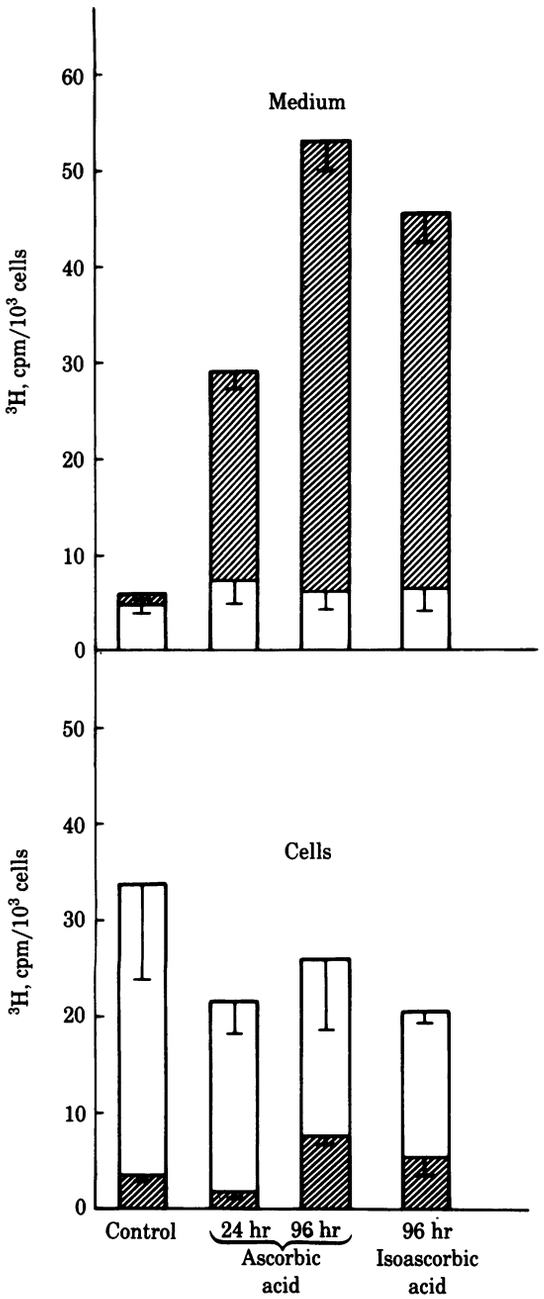

FIG. 1. Effect of ascorbate and isoascorbate on relative collagen synthesis. Human skin fibroblasts at confluent density were incubated for 4 days in Dulbecco's modified Eagle's medium supplemented with 0.5% serum. L-Ascorbic acid or D-isoascorbic acid at 0.25 mM was added before harvest for intervals of time as noted. Control cells received no additions. All cultures were labeled for the last 6 hr with L-[2,3-^{3}H]proline and L-[^{14}C]lysine. Radioactivities incorporated into collagen (hatched) and noncollagen (open) protein were determined after digestion with clostridial collagenase free from detectable nonspecific protease activity. Only ^{3}H cpm are plotted. Data represent mean ± SD for triplicate cultures. Cells were in passage 21.

corbate-deficient cultures than it did in 96-hr ascorbate-treated cultures (37% vs. 12%); nonetheless, the amount of cell pellet collagen was higher in the latter (Fig. 1). In the absence of ascorbate, collagen is underhydroxylated and would be expected to accumulate in cells. In the presence of ascorbate, secretion is enhanced and cell pellet collagen would include more insoluble extracellular collagen. In fact, large quantities of insoluble fibrillar collagen have been found to accumulate in the cell layer fractions of human lung fibroblasts grown in the presence of ascorbate (16).

The production of total noncollagen protein remained essentially unchanged under the influence of L-ascorbate or D-isoascorbate (Fig. 1). Cell growth was not affected as judged from final mean cell counts: 2.67, 2.70, 2.67, and 2.64 × 10^6 per 100-mm plate for control, 24-hr L-ascorbate-treated, 96-hr L-ascorbate-treated, and 96-hr D-isoascorbate-treated cultures, respectively.

Lysyl hydroxylase activity increased 3-fold in response to L-ascorbate or D-isoascorbate administration (Fig. 2). The stimulation was already maximal by 24 hr. Surprisingly, prolyl hydroxylase activity decreased after prolonged exposure of cells to L-ascorbate or D-isoascorbate. The apparently higher prolyl hydroxylase activity of 24-hr ascorbate-treated cultures is probably due to underestimation of the activity of untreated cultures, resulting from competition of labeled substrate in the assay with endogenous substrate. This is supported by the observation that prolyl hydroxylase activity in extracts from untreated cultures but not from ascorbate-treated cultures increased upon preincubation with cofactors for the hydroxylation reaction (data not shown).

Because treatment of cells with ascorbate resulted in changes in prolyl and lysyl hydroxylase activities, we measured the extent of hydroxylation of proline and lysine in the newly synthesized collagen. Type I procollagen was isolated from media of ascorbate-treated cultures but could not be isolated from media of untreated cultures despite repeated attempts. When collagenase-digested media proteins were examined, hydroxyproline content for untreated cultures was lower than for ascorbate-treated cultures whereas hydroxylysine content was similar (Table 1). Relative hydroxylation levels of prolyl and lysyl res-

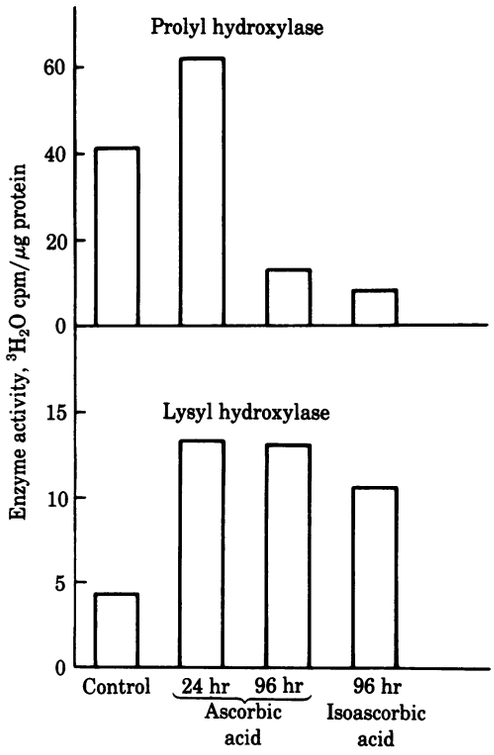

FIG. 2. Effect of ascorbate and D-isoascorbate on prolyl hydroxylase and lysyl hydroxylase levels. Details were as for Fig. 1 except cultures were not labeled. Enzyme activities in cell extracts were measured by ^{3}H release assays.

Table 1. Effect of ascorbate and D-isoascorbate on relative hydroxylation of proline and lysine in soluble extracellular collagen

Treatment	% prolyl hydroxylation*		% lysyl hydroxylation†	
	Procollagen I‡	Collagenase digest§	Procollagen I‡	Collagenase digest§
None	—	34	—	26
L-Ascorbate, 24 hr	42	47	16	25
L-Ascorbate, 96 hr	41	46	20	28
D-Isoascorbate, 96 hr	43	44	20	28

* Calculated from cpm in proline and hydroxyproline isolated in the amino acid analyzer.
† Calculated from cpm in lysine and hydroxylysine isolated in the amino acid analyzer.
‡ Isolated by DEAE-cellulose chromatography.
§ Material digested by clostridial collagenase free from detectable nonspecific proteases.

idues were similar for 24-hr and 96-hr ascorbate-treated cultures. Values for isolated procollagen were somewhat lower than for collagenase-digested material, perhaps due to additional prolyl and lysyl residues in the noncollagenous extension peptides of procollagen.

DISCUSSION

The influence of ascorbate on collagen production has been largely attributed to its role in prolyl hydroxylation (12). In the present study, collagen synthesis was assessed by measuring the incorporation of proline into collagenase-sensitive protein. The observed stimulation of collagen synthesis under the influence of ascorbate is thus independent of hydroxylation. As a corollary, it appears that, although fairly stable during growth in culture (21), phenotypic expression of fibroblasts with respect to collagen synthesis can be modified by ascorbate.

The present studies are in contrast to previous studies with human fibroblasts whose collagen synthesis either was not altered (22) or was decreased (23) by ascorbate. Whether this difference is related to cell types, experimental conditions, or method is not clear. Ascorbate has been found to have no effect on collagen synthesis in L-929 (24, 25), 3T6 (25, 26), and 3T3 (14) cells. This is not surprising because animal cell lines differ from human cell strains in many characteristics such as life-span in culture, karyotype, density dependent inhibition, and serum requirement. Human skin fibroblasts devote up to 15% of their protein synthetic capacity to collagen synthesis (17). The relatively low rate of collagen synthesis exhibited by established cell lines (25, 27) may be a consequence of the loss of ascorbate-dependent regulatory mechanism. It should be noted that collagen synthesis in transformed cells also is low and is not regulated by ascorbate (28). Ascorbate also has been found to have no effect on total collagen synthesis in human synovial cells, but this may be due to the much shorter duration of treatment used (29). In primary avian tendon cells, however, collagen synthesis was stimulated by ascorbate (15) although a later report from the same laboratory contradicted the earlier observation (30). Furthermore, it was difficult to be sure if this was a primary effect or was secondary to changes in cell density which has been found to exert a significant influence on collagen synthesis relative to noncollagen protein synthesis (17).

Most of the collagen produced under the influence of ascorbate appeared in the culture medium. The short labeling period used may have been insufficient for appreciable processing of the newly synthesized procollagen into insoluble collagen associated with the cell layer. Indeed, insoluble extracellular collagen has been found to accumulate during continuous labeling for a longer period in the presence of ascorbate (31).

The absence of detectable procollagen in medium of ascorbate-deficient cultures may be related to the instability of underhydroxylated collagen at the physiological temperature (7, 8) and consequently its susceptibility to proteolytic degradation. It is noteworthy in this regard that low molecular weight fragments presumably derived from intracellular degradation of collagen formed in the absence of ascorbate have been found in culture medium (32). However, such degradation has not been observed in other studies (26).

The collagenase-sensitive protein in medium of cultures not given ascorbate was mildly deficient in hydroxyproline. This observation is in agreement with some (23, 32) but not all (14, 16, 31, 33, 34) reports which show that collagen secreted in the absence of ascorbate is severely deficient in hydroxyproline. It is remarkable that considerable hydroxylation of prolyl residues occurred even though 0.5% dialyzed serum was used in order to minimize the contribution of serum-derived ascorbate. These results indicate the presence in cultured cells of a reducing factor that can partially replace ascorbate, as suggested earlier (24, 25).

The decline of prolyl hydroxylase activity after prolonged exposure of cells to ascorbate is unusual because changes in prolyl hydroxylase activity often accompany changes in collagen synthesis (4, 35). One report, however, indicates an adverse effect of ascorbate on prolyl hydroxylase activity with no significant change in lysyl hydroxylase activity during culture growth (36). In our experiments with growth-arrested confluent cells, lysyl hydroxylase activity was markedly stimulated by ascorbate. The significance of these observations in relation to collagen synthesis is not clear.

A comparison of short-term and long-term effects of ascorbate shows that collagen synthesis continued to increase even after prolyl hydroxylation had been maximally stimulated, suggesting that ascorbate acts at a level other than hydroxylation. This conclusion is incompatible with the postulate that intracellular accumulation of underhydroxylated collagen in ascorbate deficiency might inhibit further collagen synthesis and this inhibition could be relieved by ascorbate through hydroxylation (14). Our results raise the possibility that ascorbate may exert a more direct influence on collagen synthesis. An action of ascorbate at the level of gene expression is an attractive possibility. However, in studies with bone organ culture the stimulation of collagen synthesis under the influence of ascorbate could not be abolished by actinomycin D, suggesting a lack of effect on the synthesis of collagen mRNA (37). An alternative mechanism for ascorbate action may involve the protein synthesis machinery itself. A shift in the distribution of collagen synthesizing ribosomes toward polysomes has been observed in the presence of ascorbate (38, 39). Ultrastructural examination of normal and scorbutic tissues revealed a considerable reduction in rough endoplasmic reticulum in ascorbate deficiency, consistent with this effect of ascorbate (40).

The clinical implications of this study are appreciable. The importance of ascorbate in wound healing has been recognized for years. Ascorbate is concentrated in wounded tissues and rapidly utilized during wound healing (41–44). Tensile strength of wounds (45, 46) and incidence of wound dehiscence (47) are related to ascorbate levels. Because humans are dependent on dietary sources for ascorbate, deficiency is common in the elderly as well as sick and debilitated persons (48), who most commonly undergo surgical treatment. Such patients may need supplemental ascorbate for optimal wound healing.

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