Ascorbate Differentially Regulates Elastin and Collagen Biosynthesis in Vascular Smooth Muscle Cells and Skin Fibroblasts by Pretranslational Mechanisms*

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Ascorbate contributes to several metabolic processes including efficient hydroxylation of hydroxyproline in elastin, collagen, and proteins with collagenous domains, yet hydroxyproline in elastin has no known function. Prolyl hydroxylation is essential for efficient collagen production; in contrast, ascorbate has been shown to decrease elastin accumulation *in vitro* and to alter morphology of elastic tissues *in vivo*. Ascorbate doses that maximally stimulated collagen production (10–200 μM) antagonized elastin biosynthesis in vascular smooth muscle cells and skin fibroblasts, depending on a combination of dose and exposure time. Diminished elastin production paralleled reduced elastin mRNA levels, while collagen I and III mRNAs levels increased. We compared the stability of mRNAs for elastin and collagen I with a constitutive gene after ascorbate supplementation or withdrawal. Ascorbate decreased elastin mRNA stability, while collagen I mRNA was stabilized to a much greater extent. Ascorbate withdrawal decreased collagen I mRNA stability markedly (4.9-fold), while elastin mRNA became more stable. Transcription of elastin was reduced 72% by ascorbate exposure. Differential effects of ascorbic acid on collagen I and elastin mRNA abundance result from the combined, marked stabilization of collagen mRNA, the lesser stability of elastin mRNA, and the significant repression of elastin gene transcription.

Ascorbate, along with ferrous ion and α-ketoglutarate, is a cofactor for the enzymatic activity of prolyl hydroxylase, a heteromer that hydroxylates prolyl residues in procollagen, elastin, and other proteins with collagenous domains prior to triple helix formation (1–6). Ascorbate in low concentrations is essential for production of collagen, since a minimum of 35% of the prolyl residues in collagen need to be hydroxylated for the collagen molecule to maintain its triple-helical conformation at physiologic temperatures (3). Ascorbate is also a cofactor for lysyl hydroxylase (7). Further modification of hydroxylysine has key effects on collagen fiber organization (8).

In addition to its direct, rapid effect on hydroxylation, ascorbate, at levels approaching 50 μM, has been found to cause a 6-fold increase in the rate constant for procollagen secretion (9), as well as an increase in collagen gene transcription and collagen mRNA levels in various cell strains (9–17). This suggests that ascorbate action not only involves hydroxylation and stabilization of the triple helix, it also involves direct or indirect effects on gene expression and protein secretion. The increase of type I collagen production in cells cultured in the presence of ascorbic acid is well known (5, 15, 18–23) and has been investigated extensively; however, the effects of ascorbate on other extracellular matrix molecules are still poorly understood. Several studies have shown that vitamin C exerts a negative effect on elastin accumulation (5, 24–28). Although hydroxyproline is a normal, minor constituent of insoluble elastin, it has been suggested that ascorbate might impair elastin production by overhydroxylation of its prolyl residues (24, 29, 30). However, elastin secretion is hydroxylation-independent (31).

The aims of our study were to investigate, in two different cell culture models: (a) the time- and dose-dependent effect of ascorbate on type I collagen and elastin production and mRNA expression, and (b) evidence for these effects being due to a pre- and/or posttranslational mechanism of regulation. The possible effect of the redox properties of this vitamin on connective tissue metabolism is discussed.

MATERIALS AND METHODS

Cell Culture—Pig aortic and pulmonary artery smooth muscle cells and pig skin fibroblasts were obtained from newborn to 14-day-old domestic pigs sacrificed by anesthesia and exsanguination. Tissues were removed and placed immediately on ice in transport medium consisting of Dulbecco's modified Eagle's medium (DMEM)[1] containing 5% fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 1000 units/ml penicillin, 1000 μg/ml streptomycin, 2.5 μl/ml fungizone (Life Technologies, Inc.), 0.03% glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Life Technologies, Inc.). Using sterile technique, descending aortas from several 1-day-old pigs were stripped of the external, adventitial layer and dissected into four serial segments, comparable in length, designated as A, B, C, and D as described previously (32). Tissues were pooled according to segments, finely minced, and incubated with 200 units of crude collagenase (type IA, Sigma) in transport medium for 4–6 h at 37 °C. Digested tissue was washed to remove excess collagenase, and cells were allowed to migrate

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; TE, tropoelastin; PBS, phosphate-buffered saline; mRNA$_E$, elastin mRNA; kb, kilobase(s); SMC, smooth muscle cell(s).

from explants in plastic tissue culture dishes (Corning, Marietta, GA) in DMEM, 20% FCS and antibiotics and were maintained in 5% CO_2, 95% humidified air at 37 °C. Pig pulmonary arteries (divided in inner and outer medial layers), pig skin, and human skin biopsies were cultivated by outgrowth as described (33).

To assure an elastogenic phenotype, smooth muscle cells were used at passage 2–3, in triplicate cultures. Ascorbate, glutathione, and dehydroascorbic acid were dissolved in sterile H_2O and stored at −20 °C until use. Pig skin fibroblasts used for *in situ* hybridization, at passage 3, were subcultivated in triplicate in eight-well culture chamber slides (1 cm²/well; Lab-Tek, Nunc Inc., Naperville, IL) and allowed to reach confluence in DMEM, 20% FCS. At confluence, cells were fed with medium containing 10% newborn calf serum supplemented with 0, 1, 10, or 50 μg/ml ascorbate, and after 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 h, they were fixed in 4% paraformaldehyde (Fluka; Ref. 34) in phosphate-buffered saline (PBS) plus 5 mM $MgCl_2$ at 4 °C for 2 h, washed in PBS, dehydrated in graded ethanol, and stored in 80% ethanol at 4 °C. Prior to hybridization, cells were hydrated and treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8, for 10 min at 25 °C, followed by 50% formamide in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7) for 10 min at 60 °C.

Quantitation of Soluble Collagen and Elastin—A standard ELISA was used to evaluate both type I procollagen and tropoelastin (TE) production in media samples from the same cell populations (35, 36). Type I procollagen production was measured in 48-h media using rabbit antiserum raised against the triple-helical portion of native, porcine, type I collagen. The IgG fraction from this antiserum showed no cross-reactivity with porcine type III or type V collagens (35). Native type I collagen was used for coating the plates and as the competing antigen in order to generate a standard curve.

TE production in pig smooth muscle cells and pig fibroblasts was evaluated in 48-h medium using rabbit antiserum to pig α-elastin at a 1:2000 dilution. Pig α-elastin (40 ng/well) was used as the adsorbing antigen, and pig TE (0.17–22 ng) was used for constructing a standard curve. The same conditions were used for elastin quantitation in cultured human fibroblasts, except that the coating antigen was human α-elastin. Data for both type I collagen and TE production were calculated on a BASIC microcomputer program (37) or analyzed by MacReader software obtained from Bio-Rad. All values were converted to molecular equivalents per cell per hour, assuming a molecular mass of 285,000 daltons for type I collagen (the standard used), and 70,000 daltons for TE.

DNA Quantitation—Cells were washed twice with PBS, and DNA assays were performed in triplicate using a fluorimetric assay (38).

Elastin and Collagen mRNA Isolation and Quantitation—RNA was isolated from aortic smooth muscle cells using guanidine HCl/CsCl fractionation as described previously (39). RNA from pulmonary artery smooth muscle cells was extracted in acid phenol/chloroform (40). Equivalent amounts of RNA (2 μg) were examined for the presence of specific type I collagen and TE transcript by dot blot hybridization onto nitrocellulose (41). RNA from each sample was resuspended in denaturing buffer (containing 3 parts 20 × SSC, 2 parts 37% formaldehyde, and 6 parts 10 mM Tris, 1 mM EDTA, pH 8.0) and then serially diluted at a 1:2 ratio in 15 × SSC in a siliconized, 96-well microtiter plate. Samples were dot-blotted onto nitrocellulose using the Life Technologies, Inc. 96-well Hybridot apparatus, and then the nitrocellulose was baked at 80 °C under partial vacuum for 2 h. The nitrocellulose was then prehybridized overnight in a solution containing 5 × SSC, 0.1% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution (1 × Denhardt's: 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 0.1 M sodium phosphate pH 6.7, 250 μg/ml herring sperm DNA, and 50% formamide. Hybridization was done at 45 °C for 24 h using fresh solution containing nick-translated ³²P-labeled probes to a specific activity of 0.5–1 × 10⁸ cpm/μg, followed by 30-min washes to a final stringency of 0.2 × SSC, 0.1% SDS, at 60 °C. Type I collagen mRNA was detected using HF1131, a 1.9-kb collagen type I cDNA insert in the *Eco*RI site of pBR322 corresponding to the COOH-terminal portion of the pro-α2(I) chain of human procollagen. mRNA$_E$ was detected with a sheep elastin genomic DNA probe, pSE1–1.3, containing the carboxyl-terminal exon and about two thirds of the long, 3′-untranslated portion of the sheep elastin mRNA (43). Autoradiography was done on preflashed Kodak X-AR film with intensifying screens. Quantitation of specific mRNA was done by scanning densitometry on a Helena densitometer.

In Situ Hybridization—The following probes were used: (*a*) the 1.9-kb human COL1A2 probe HF1131 (42); (*b*) cHE-4, a 1.0-kb human elastin cDNA fragment cleaved with *Bam*HI and *Hin*dIII from a larger insert in the *Eco*RI site of Bluescript plasmid (Stratagene, La Jolla, CA)

corresponding to exons 18–36 of human elastin (44); (*c*) pHcIII-I, a 1.9-kb collagen type III cDNA insert in the *Pst*I site of pGEM-4Z (Promega, Madison, WN) corresponding to the α1 chain of type III collagen (45); (*d*) pFN771, a 1.0-kb fibronectin cDNA fragment in the *Eco*RI site of pBR322 corresponding to part of the fibrin-binding domain and the 3′-noncoding region of human cellular fibronectin (46); (*e*) pGM4TRHB, a 550-base pair rat stromelysin-1 cDNA insert in the *Bam*HI and *Hin*dIII sites of pGEM-4Z, corresponding to a portion of stromelysin located 440 base pairs from its 3′ end (47). The probes were labeled with [³⁵S]dCTP (DuPont NEN) by random priming (48, 49) to a specific activity of 0.5–1.5 × 10⁸ dpm/μg of DNA. The hybridization mixture, containing the labeled probe (10,000 dpm/μl), 100 μg/ml salmon sperm DNA (Sigma), 100 μg/ml yeast tRNA (Sigma), 30–50% formamide (Fisher), 12.5% dextran sulfate (Pharmacia Biotech Inc.), 0.01 M dithiothreitol (Sigma) in Denhardt's solution, was heated at 90 °C for 5 min and then chilled on ice for other 5 min. 60 μl of the solution were spread on each slide, then covered with siliconized coverslips and incubate for 15–18 h at 37 °C to 42 °C in a water bath. Slides were washed in two changes of Denhardt's solution plus 0.01 M dithiothreitol and 30% formamide, and then in decreasing concentrations of SSC to 0.1 × SSC at 45 °C followed by dehydration. The slides were dipped in Kodak NTB2 emulsion, boxed for 7 days at 4 °C, and then developed in Kodak D19 developer and fixed at 15 °C. Cells were stained with hematoxylin and eosin, observed in bright and dark field with an Olympus BH-2 photomicroscope and photographed with PanatomicX film.

mRNA Stability—Pig pulmonary artery smooth muscle cells were grown to confluence in 100-mm tissue culture dishes in DMEM, 10% FCS, followed by exposure for 24 h to two daily doses of 50 μg/ml ascorbate. Medium was changed and one half of the cultures were maintained in 50 μg/ml ascorbate, added twice daily, while ascorbate was withdrawn from the remainder. Half of each set received 7 μg/ml actinomycin D (1 mg/ml in 95% EtOH; Sigma). At 0, 2, 4, 8, 12, and 24 h, cells were washed three times in ice-cold PBS and lysed in 2 ml of 4 M guanidine isothiocyanate containing 0.1 mM β-mercaptoethanol. DNA was sheared by passage three times through a 22-gauge needle, and RNA was isolated by extraction in acid phenol/chloroform (40). For Northern hybridization, 9 μg of total RNA was denatured in 50% formamide, 1 M formaldehyde at 68 °C for 10 min and separated by electrophoresis through a 1.2% agarose gel containing 1 M formaldehyde. RNA was transferred by capillary action with 6 × SSC to nylon membranes supplied by Micron Separations Inc., Westboro, MA, and baked for 2 h *in vacuo* at 80 °C. The RNA molecules were hybridized to 30 ng of three DNA fragments: the elastin probe, HDE3 (50); the 1.9-kb human COL1A2 probe, HF1131 (42); and a cyclophilin probe, 1B15 (51), each labeled by random priming according to instructions from the manufacturer (Stratagene, La Jolla, CA). The filters were washed once for 30 min in 1 mM EDTA, 40 mM Na_2PO_4, pH 7.2, 5% SDS at 65 °C; and twice for 30 min in 1 mM EDTA, 40 mM Na_2PO_4, 1% SDS at 65 °C. Autoradiography to detect hybridizable transcripts was for 2 days in cassettes with an intensifying screen. The optical densities of autoradiographic signals for each transcript were normalized to those for 1B15, and the transcription values were calculated as the ratio of each transcript signal relative to the constitutive, 1B15 signal, or to each other.

Nuclear Run-on Transcription—Smooth muscle cells from porcine pulmonary artery were subcultivated in two 150-mm dishes (Corning) and fed with DMEM containing 10% FCS (Atlanta Biological). At confluence one dish was treated with a total of 150 μg/ml L-ascorbic acid, in two divided, daily doses, for a total period of 72 h. The untreated dish was used as a control and was maintained in the same culture conditions. Nuclei were isolated from cells and mixed with 100 μl of reaction buffer and 100 μCi of [α-³²P]UTP (DuPont NEN) as described previously (52). Slots on a nylon membrane (Magna Graph MSI, Westboro, MA) contained 10 or 15 μg of cDNA inserts for human elastin (HDE-3; Ref. 44), human COLIA2 (42), and the cyclophilin probe 1B15 (51). DNA was immobilized by baking 2 h at 80 °C and by UV cross-linking. The filter was subsequently hybridized, washed, and exposed to a Phosphor-Imager plate (Molecular Dynamics, Sunnyvale, CA). The optical densities of autoradiographic signals were normalized to that for cyclophilin, and the transcription values were calculated as the ratio of each transcript signal relative to the cyclophilin one.

RESULTS

Effects of Ascorbate on Collagen and Elastin Production— Preconfluent monolayers of porcine vascular smooth muscle cells (SMC) were continuously exposed to 50 μg/ml ascorbate, a
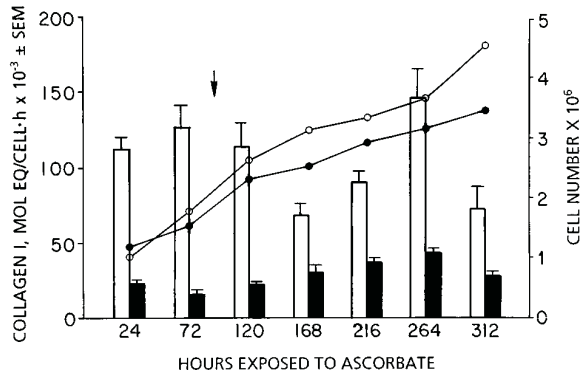
FIG. 1. **Type I collagen production and cell proliferation are stimulated in pig aortic smooth muscle cells at low and high cell density.** Second passage, preconfluent smooth muscle cells from a 1-day-old pig were supplemented with daily doses of 50 μg/ml ascorbate. Collagen production over 48-h intervals was evaluated by ELISA of the culture medium. Production data are expressed as molecular equivalents/cell h $\pm$ S.E. of collagen type I produced by triplicate cultures grown in the presence (*open bars*) or absence (*solid bars*) of ascorbate. *Open* and *closed circles* represent cell numbers from ascorbate-treated and ascorbate-deficient cultures, respectively. The *arrow* indicates the time at which cells reached visual confluence.

FIG. 2. **Ascorbate inhibits tropoelastin production particularly in postconfluent pig aortic smooth muscle cells.** Preconfluent SMC at second passage were exposed daily to 50 μg/ml ascorbate, as described in Fig. 1. Tropoelastin production was evaluated by ELISA in medium accumulated over 48-h intervals and expressed as molecular equivalents/cell·h $\pm$ S.E. *Open* and *closed bars* represent cells grown in the presence or absence of ascorbate, respectively. Cell number data are identical to those of Fig. 1.

concentration that maximally stimulates collagen production, with addition of fresh ascorbate every 24 h. Less frequent addition did not produce consistent effects (data not shown). As shown in Fig. 1, the expected stimulatory effect on collagen I production in these cultures was independent of cell density. From the time of treatment (3 days before confluence) until the termination of the experiment, collagen production was amplified from 2- to 8-fold by treatment with vitamin C. Under scorbutic conditions, we observed a small, significant rise in collagen production in postconfluent (>72 h) cultures. Since smooth muscle cells continue to overgrow after reaching confluence, both ascorbate supplemented and deficient cultures showed a continuous increase in cell number. Ascorbate-treated cultures grew at a slightly faster rate. In contrast to the positive effects on collagen production, tropoelastin production was markedly diminished by ascorbate treatment (Fig. 2). As shown previously (36), tropoelastin production under control conditions did not approach a maximum until cells reached confluence (>72 h). Thus, the most significant inhibition by ascorbate was seen in postconfluent SMC cultures. Tropoelastin production on different days of culture ranged from 30% to 50% of untreated cultures.

As suggested from the foregoing data, ascorbate effects depended on the differentiation state of the target cell. In one experiment, we compared ascorbate inhibition in thoracic *versus* abdominal aortic SMC. Previous experiments have shown marked, site-specific differences in basal production of tropoelastin in this tissue (32, 33). Fig. 3 illustrates the 2–3-fold higher basal production levels in SMC from thoracic *versus* abdominal aortic segments of the newborn pig. When SMC from each of these segments were treated with ascorbate, the relative inhibition in the thoracic segments was far greater than in the abdominal segments.

Ascorbate inhibition of tropoelastin production showed a dose-dependent relationship (Fig. 4). Inhibition was observed in some experiments at doses as low as 0.5 μg/ml ascorbate, and the degree of inhibition was significant at 5–10 μg/ml. Effects of ascorbate were not immediate. Fig. 5 illustrates the time and dose-dependent effects of ascorbate on a different cell population, human skin fibroblasts. In this experiment, it was observed that daily addition of at least 60 μg/ml ascorbate was required to obtain significant inhibition, and exposure to the vitamin for 3 days was necessary at this dose. At higher doses
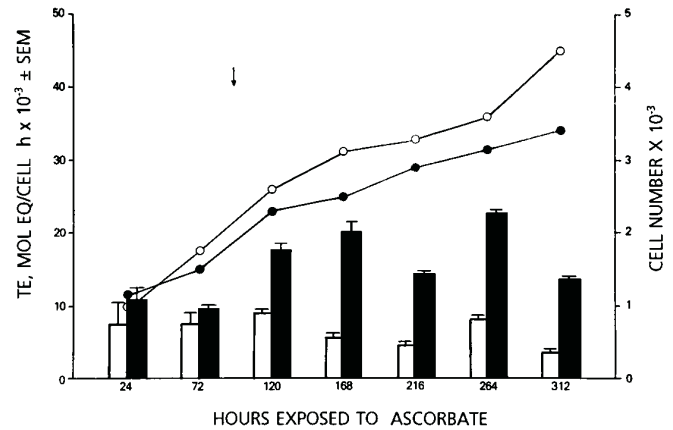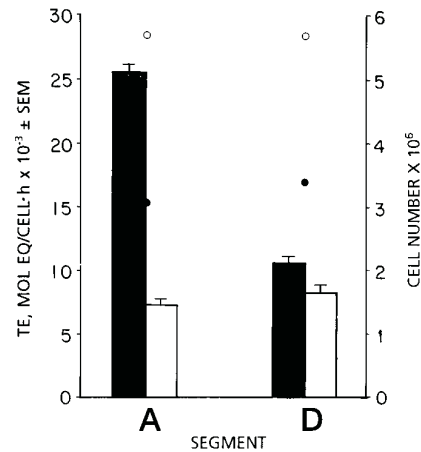

FIG. 3. **Segmental variation in the effect of ascorbate on tropoelastin production.** Pig smooth muscle cells derived from thoracic (*A*) and abdominal (*D*) segments of a newborn porcine aorta were maintained in 50 μg/ml·day ascorbate. Culture medium was changed every 3 days. At confluence media were changed and harvested 48 h later for evaluation of elastin production, which is expressed as molecular equivalent/cell·h $\pm$ S.E. *Solid bars*, TE production $-$ ascorbate; *open bars*, TE production $+$ ascorbate; *open circles*, cell number $+$ ascorbate, *closed circles*, cell number $-$ ascorbate.

(100 μg/ml) inhibition was more immediate and more extensive. In many subsequent experiments, we used a protocol in which ascorbate was added at lower, divided doses in an effort to maintain a more constant, biologically active concentration. This treatment regime produced the most consistent and effective inhibition of TE production.

To help confirm the specificity of the ascorbate effect, confluent, thoracic SMC were incubated with another reducing agent, glutathione (GSH; 0.28–3.0 mM), and an inactive form of ascorbate, dehydroascorbic acid (0.28 mM). At levels up to 1.0 mM, glutathione did not reduce TE production, while at a 10-fold molar excess relative to ascorbate (3.0 mM), there was a partial diminution of TE production (Fig. 6). The latter effect was attributable to cytotoxicity of the compound (data not shown). Dehydroascorbate treatment showed no significant effect on TE production, while ascorbate at an equivalent concentration produced a ~60% reduction in TE production.

Ascorbate Reduces Elastin Production by Pretranslational Mechanisms—Messenger RNA levels for collagen I and elastin
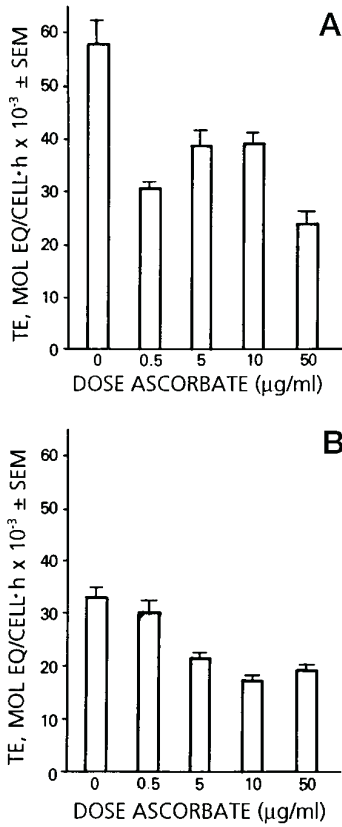
FIG. 4. **Dose-dependent inhibition of tropoelastin production by ascorbate.** Pig smooth muscle cells derived from segment A (*panel A*) and B (*panel B*) of a 1-day-old pig were exposed daily to increasing amounts of ascorbate. Tropoelastin production was evaluated by ELISA and expressed as molecular equivalents/cell·h $\pm$ S.E.
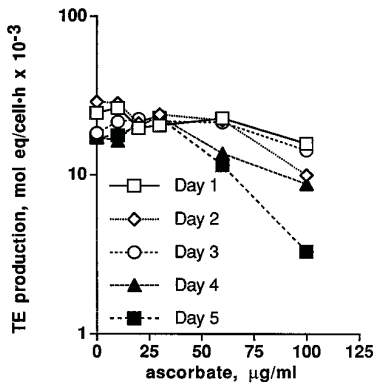

FIG. 5. **Dose- and time-dependent reduction of elastin production in normal human skin fibroblasts by ascorbate.** Skin fibroblasts were grown to confluence and pre-exposed for 1–5 days, on a daily basis, to ascorbate (10–100 μg/ml), in the presence of 5% newborn calf serum. The media were changed, maintaining the same ascorbate and serum concentrations, and elastin accumulation in culture media was evaluated over an additional 48 h. Data are expressed as molecular equivalents/cell·h.

were evaluated subjectively by *in situ* hybridization of skin fibroblasts grown in multiwell culture slides in the presence of varying concentrations of ascorbate (Fig. 7). Under these culture conditions, a 24-h exposure was sufficient to alter markedly the relative abundance of these two transcripts. In Fig. 7, the *upper panel* shows that ascorbate was able to cause dose-dependent increases in collagen I transcript levels, while the *lower panel* shows a dose-dependent inhibition of elastin mRNA under the same culture conditions. In sister cultures, ascorbate was effective in increasing COL3A1 mRNA levels,
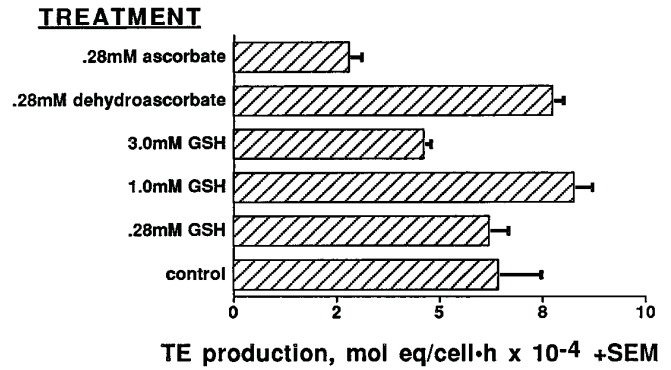

FIG. 6. **Specificity of reduced ascorbate inhibition of elastin production.** Porcine SMC from the thoracic segment were grown to preconfluence and exposed to the reducing agents ascorbate and glutathione, as well as to an inactive form of ascorbate, dehydroascorbic acid, at the indicated doses. Elastin production in the media was evaluated after 48 h of incubation and expressed as molecular equivalents/cell·h $\pm$ S.E.
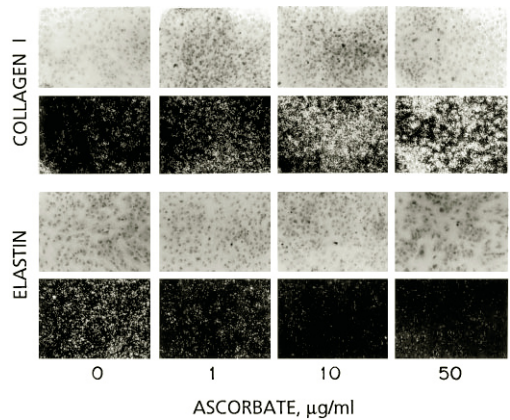

FIG. 7. **Differential expression of elastin and collagen mRNA levels in pig skin fibroblasts by *in situ* hybridization.** Confluent cultures of porcine skin fibroblasts were exposed to increasing amounts of ascorbate (0–50 μg/ml) for 24 h and processed for *in situ* hybridization. Cells were stained with hematoxylin and eosin after emulsion development. Brightfield (*top*) and darkfield (*bottom*) images of *in situ* hybridization reactions for collagen and elastin, respectively, are illustrated. Original magnification, ×33.

while no remarkable changes were seen in mRNA levels for fibronectin and stromelysin (data not shown). Reduction of $mRNA_E$ by increasing ascorbate dose was quantified by dot blot hybridization and Northern blot analysis; elastin mRNA steady state levels in the presence of 10 and 50 μg/ml ascorbate were 30–40% of that seen without ascorbate. Fig. 8 shows the kinetics of $mRNA_E$ reduction when porcine pulmonary aorta SMC were exposed to two daily additions of 50 μg/ml ascorbate over a 48-h period. The data, normalized to the expression of a constitutive transcript (cytochrome oxidase II; Refs. 53 and 54), showed that elastin mRNA under these circumstances was reduced to less than 20% of control values.

Since several factors modulate elastin transcript levels by altering mRNA stability (55), we evaluated the stabilities of elastin and collagen I mRNAs under conditions of continuous ascorbate exposure and upon withdrawal of ascorbate. Smooth muscle cultures that had been previously treated with ascorbate (2×50 μg/ml·day) were changed to medium lacking ascorbate and containing actinomycin D to block transcription. When ascorbate was removed from the cultures, there was an increase in the apparent $t_{1/2}$ of $mRNA_E$ from approximately 19 h to 33 h (Fig. 9, *panel A*). This stability difference was selective, since $mRNA_E$ was less stable than a constitutive gene, cyclophilin, only in the presence of ascorbate (Fig. 9, *panel B*).
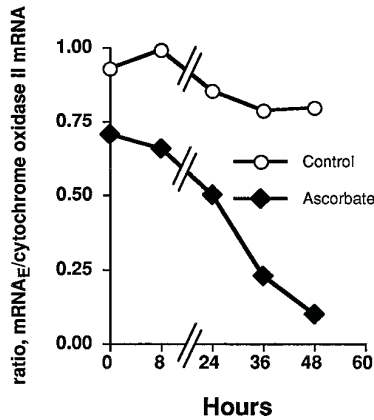
FIG. 8. **Kinetics of mRNA$_E$ reduction by ascorbate.** Smooth muscle cells from the pulmonary aorta of a 6-month-old pig were grown in 100-mm tissue culture dishes. At confluence cells were fed with DMEM, 5% newborn calf serum and supplemented with three daily additions of 50 μg/ml ascorbate (*solid symbols*) or nothing (*open symbols*). RNA was isolated from cells at indicated times and identified by Northern blot hybridization with an elastin cDNA probe. Densitometric analysis of autoradiographic images of mRNA$_E$ were normalized to the intensity of the constitutive probe, cytochrome oxidase II.

Conversely, COL1A2 transcripts were markedly destabilized by ascorbate withdrawal ($t_{1/2} \approx 7$ h *versus* 32 h; Fig. 9, *panel C*). Because of the inverse behavior of the two transcripts, ascorbate depletion caused rapid rise in the ratio of ELN to COL1A2 transcripts during the first 12 h after ascorbate withdrawal (Fig. 9, *panel D*, *open circles*), while in the presence of ascorbate the proportion of mRNA$_E$ to COL1A2 mRNA progressively declined (Fig. 9, *panel D*, *solid squares*).

These studies using a transcription inhibitor suggested that reduced mRNA$_E$ stability was insufficient to account fully for reduced mRNA$_E$ levels in ascorbate-treated cells, while changes in COL1A2 transcript stability were certainly correlated with collagen I biosynthesis and mRNA levels. We therefore performed nuclear run-on transcription studies to determine further the mechanisms that brought about reduced mRNA$_E$ levels. Cells were exposed to ascorbate for 48 h, and elastin and collagen transcription rates were evaluated relative to that of cyclophilin. As shown in Fig. 10, the ELN transcription rate was reduced by 70%, while the COL1A2 transcription rate was not significantly altered. Taken together, these data suggest that elastin mRNA abundance was predominantly affected by ascorbate at a transcriptional level with a lesser, significant effect on mRNA$_E$ stability, while the collagen mRNA increase was predominantly controlled, at least in these cells, at a posttranscriptional level.

DISCUSSION

The requirement for ascorbic acid to maintain connective tissue integrity was observed centuries ago by Lind (56). Subsequently, ascorbate was found to play a role in several physiologic systems (57, 58). With modern appreciation of the primary structure of collagen came the understanding that hydroxyproline played a crucial role in stabilizing the collagen triple helix. *In vitro* and *in vivo* studies indicated that ascorbic acid played a role in the generation of this important posttranslational modification, and detailed biochemical investigation revealed the precise role of ascorbate during the transfer of molecular oxygen to prolyl (and lysyl) residues (59). The observation that elastin also contained a significant amount of hydroxyproline led a number of investigators to examine its role in the biosynthesis of elastin. Using proline analogs or hypoxic culture conditions, these investigators demonstrated that prolyl hydroxylation was not required for the biosynthesis and
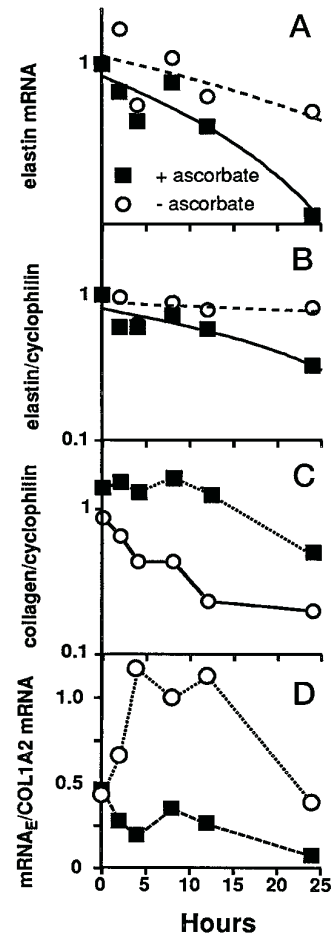

FIG. 9. **Effect of ascorbate withdrawal on stabilities of elastin and collagen mRNA.** Cultures of porcine SMC were pretreated for 24 h with 2 doses of 50 μg/ml ascorbate. Actinomycin D (7 μg/ml) was added at time = 0, and ascorbate was maintained or removed. *Solid squares*, ascorbate maintenance; *open circles*, ascorbate withdrawal. mRNA levels were quantified by scanning densitometry of Northern blots hybridized to ELN, COL1A2, and cyclophilin probes. *Panel A*, effect of ascorbate withdrawal on elastin mRNA stability. Hybridization signals for elastin mRNA in the presence and absence are expressed in relative optical density units on a logarithmic scale. Data are normalized for differences in input RNA. Linear regression curves with and without ascorbate present showed differing apparent decay rates, although the scatter of the data yielded slopes that were not statistically different ($0.1 > p > 0.05$). The estimated $t_{1/2}$ was ~32 h in the absence and ~19 h in the presence of ascorbate. *Panel B*, elastin mRNA stability normalized to a constitutive gene, cyclophilin. To correct for possible loading differences and for general effects, signals for mRNA$_E$ were related those of cyclophilin and normalized to unity at the start of actinomycin D addition and ascorbate withdrawal (0 h). Data are on a logarithmic scale. *Panel C*, effect of ascorbate withdrawal on collagen mRNA stability. COL1A2 levels were normalized to the constitutive gene, cyclophilin, and plotted on a logarithmic scale. Collagen mRNA was markedly destabilized by ascorbate withdrawal. *Panel D*, elastin mRNA is relatively more stable than collagen I mRNA upon ascorbate withdrawal. Experimental data compare the ratio of elastin mRNA to collagen I α2 chain mRNA as a function of time after addition of actinomycin D ($t = 0$) and maintenance or withdrawal of ascorbate. Elastin mRNA was much less sensitive to ascorbate withdrawal than collagen I, thus maintaining a higher ratio. In the continued presence of ascorbate, mRNA$_E$ was slightly less stable than collagen I mRNA, causing a fall in the ratio.

secretion of elastin, suggesting that hydroxyproline either played a different role in elastin or was produced as a byproduct of the coincident synthesis of collagen and elastin by many types of cells (30, 31, 60). In contrast, the findings of de Clerck and Jones (5) and Scott-Burden *et al.* (28) suggested that ascorbate, at concentrations that maximally stimulated collagen biosynthesis, was actually an antagonist of elastin accumula-
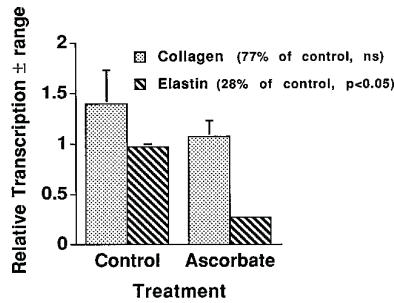
FIG. 10. **Transcriptional effects of ascorbate.** COL1A2, cyclophilin, and ELN cDNAs were bound at several concentrations onto a nylon filter and hybridized with the products of nuclear run-off assays from cells treated with ascorbate (75 μg/ml) twice daily for 3 days. Signal strengths for COL1A2 and ELN were quantified by phosphoimage analysis and normalized to the signal derived from a constitutive probe, cyclophilin, at different DNA concentrations. Data represent the means of two determinations $\pm$ S.E. Where not plotted, the range was too small to visualize. Significance was estimated by Student's *t* test.

tion. In a series of studies, Franzblau and co-workers (25–27) elaborated on these observations by showing that elastin accumulation was sharply diminished in cell cultures treated with vitamin C. To extend these observations to a more mechanistic level, we asked whether ascorbate had effects on elastin biosynthesis, and if so, whether these biosynthetic effects were due to changes in availability of elastin mRNA. The present studies demonstrate that ascorbate reduces synthesis of elastin in cultured cells by diminishing the abundance of elastin gene transcripts, and that the reduction in elastin mRNA is due to contribution from altered transcript stability and reduced transcription.

In our investigation, we demonstrated that the previous observations made both *in vivo* and *in vitro* on the effects of high levels of ascorbate on elastin accumulation were attributable to reduced biosynthesis. The data show that the ascorbate levels used in this study, those typically used to maximize collagen production, generated opposing patterns of biosynthesis of the two matrix proteins, type I collagen and elastin. Effects of vitamin C on elastin production in cell culture depended on both dose and time of exposure. The fact that lower doses of ascorbate appeared to require longer exposure times may be related to the rapid oxidation of ascorbic acid under standard culture conditions. Although biological effects could be reproducibly observed with daily doses of ascorbate, more effective or dramatic effects on elastin biosynthesis were obtained when the vitamin was added as two to three divided doses every day. Recently, investigators have reported a novel form of ascorbate, ascorbate 2-phosphate, that appears to have a much longer biological half-life (16, 17). Preliminary findings confirm that using the more stable form of ascorbate, long exposure times and multiple doses are not necessary to obtain significant reduction of TE production.[2]

Bergethon *et al.* (25) showed that ascorbate and isoascorbate had equivalent effects on elastin accumulation and elastin prolyl hydroxylation, while another reducing agent, dithiothreitol, had no effect. The effect of ascorbate appeared to be specific to the active form of the molecule. Our present findings show that another physiologic reducing agent, glutathione, did not have an effect on elastin production except at toxic levels, and dehydroascorbate, which also has reducing potential but lacks prolyl hydroxylase cofactor activity, did not affect elastin production in cultured cells.

The effect of ascorbate on elastin production did depend on the state of cell differentiation. Inhibition was less evident in

cells at low density and in a phase of rapid growth, a phase at which relatively low levels of elastin production are observed. As cells neared confluence (postconfluence in the case of smooth muscle cells), higher levels of elastin production developed, and ascorbate inhibition reached its maximal extent. Likewise, the effect of ascorbate on the more elastogenic thoracic aortic smooth muscle cells was much greater than in abdominal aortic smooth muscle cells. Although ascorbate had small effects on cell proliferation, as also reported by others (61), these changes were not sufficient to account for changes in elastin expression, and all data were normalized to cell number.

There did not appear to be any heterogeneity to the ascorbate response, at least in fibroblast populations that were examined by *in situ* hybridization. Changes in mRNA levels appeared to be uniform throughout the culture population. *In vivo*, the effects of excess ascorbate appear to be most prominent in the vessel wall (62), which may reflect the high state of elastogenesis. Similarly, supravalvular aortic stenosis appears to arise from the rate-limiting production of elastin in the aortic root due to a large truncation of one elastin allele (63).

Many previous studies on the effect of ascorbate on elastin did not discriminate between an effect upon synthesis or accumulation. One exception is the recent report that short term (48 h), low dose ascorbate (10 μg/ml) appears to increase the stability of secreted tropoelastin without an effect on mRNA levels (64). Overhydroxylation of tropoelastin may affect its turnover or incorporation into the extracellular matrix. The present studies show that most of the long term effects of ascorbate can be ascribed to biosynthesis. Given this observation, we wished to know whether the regulation was a pre- or posttranslational event. Studies of the secretory pathway of elastin had suggested that impaired secretion might lead to a form of negative feedback regulation at the transcript level (65). It has also been proposed (25) that the overhydroxylation of elastin, which might occur in ascorbate-supplemented conditions, could alter the rate of secretion as a result of conformational alterations that affect secretion and that ultimately feed back to down-regulate mRNA$_E$ levels. In addition, there is experimental evidence that the presence of hydroxyproline in typical elastin primary sequences alters its thermodynamic behavior (66). Investigations of the effect of ascorbate on collagen I transcript levels have shown regulation at the level of both mRNA stability and transcription (12). Although the majority of studies, using acute ascorbate treatment, show substantial transcriptional activation (15, 17, 67), our own data emphasize the role of mRNA stability. The prolonged exposure to ascorbate may attenuate the transcriptional response. The mechanisms for altering collagen transcript stability are unknown (12), and a number of pathways have been proposed to explain the effect of ascorbate on transcriptional activity (68). A recent hypothesis has invoked the ability of ascorbate to generate lipoperoxides, which produce reactive aldehydes that could act upon the transcriptional machinery (67, 69–71). For example, malondialdehyde has been shown in one study to modulate the production of collagen in fibroblasts (67). This area of investigation is controversial, since a very recent report has shown that cell-impermeant agents such as desferroxamine and EDTA can block the production of lipoperoxides without affecting the ability of ascorbate to stimulate collagen production (72). It is not known whether lipoperoxidation mediates the effects of ascorbate on elastin production.

Elastin mRNA abundance is regulated by both transcription and mRNA stability (55, 73). Transcriptional regulators include insulin-like growth factor-1 (+), tumor necrosis factor-α (−), transforming growth factor-β (+), retinoic acid (+), and

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[2] O. Zoia and J. M. Davidson, unpublished observation.

interleukin-1 (−). $mRNA_E$ stability is strongly modulated by agents that affect protein kinase C (phorbol esters), transforming growth factor-β, and 1,25-dihydroxyvitamin D_3 (55). Since mRNA stability is a major factor in elastin regulation, it was reasonable to ask whether the vitamin had an effect on elastin mRNA stability. As the biosynthetic responses of collagen I and elastin are opposite, we could readily determine whether their stabilities were also inversely affected by ascorbate treatment. Under conditions of ascorbate withdrawal, COL1A2 mRNA was degraded more rapidly than $mRNA_E$, while under conditions of ascorbate supplementation, COL1A2 mRNA was stabilized markedly ($t_{1/2}$ increased 4.9-fold, from 7 to 33 h) as $mRNA_E$ stability declined by a factor of 2. Since the concentration of $mRNA_E$ fell more rapidly in the presence of continuous ascorbate than predicted by the stability study, it is likely that the transcription of $mRNA_E$ is the dominant site for the effect of ascorbate supplementation. Nuclear run-on transcription confirmed this concept, and the inhibition of ELN transcription by ascorbate was very similar in magnitude to the reduction of $mRNA_E$ concentration and elastin production. The present experiments confirmed many reports of increased collagen mRNA levels after ascorbate treatment (15, 16, 68, 74), but they failed to corroborate the 2–4-fold increases in transcription observed in a number of cases (16, 17, 75). The transcriptional studies have used skin fibroblasts, and it is conceivable that regulation differs in elastogenic, vascular smooth muscle cells. It is also possible that the ascorbate doses that maximally inhibit elastin expression, while stimulating collagen mRNA accumulation, are suboptimal for stimulation of collagen transcription. Using estimates of a 4.9-fold increase in COL1A2 stability (32.5 h *versus* 6.7 h) and a 2.8-fold relative decrease in ELN transcription (0.78 for COL1A2 *versus* 0.28 for ELN), the kinetics predict a 3.8-fold increase in COL1A2 mRNA concentration (4.9 × 0.78) and a 3.6-fold decrease in elastin mRNA concentration in the presence of ascorbate. The net difference (COL1A2/ELN) would be a 13.7-fold relative difference in expression. These mRNA dynamics are highly consistent with the observed changes in both steady state mRNA levels and protein production in smooth muscle cells treated with vitamin C.

Yet to be established is the mechanism by which ascorbate, an essential cofactor for prolyl and lysyl hydroxylation and an ubiquitous reducing agent, bring about changes in elastin gene transcription. At present, we cannot exclude the concept that lipoperoxides play a role in the metabolic pathway (23, 67, 71, 72). Preliminary data would suggest that the ascorbate effect on elastin in cell culture can be modulated by the presence of free radical scavengers such as α-tocopherol and by oxygen concentration.[2] Even if the effect of ascorbate is at the level of ELN transcription, there remains the intriguing possibility that these presumed transcriptional effects, which take a substantial number of hours or even days to appear, are secondary to effects on the secretory pathway or to the accentuated, pericellular accumulation of collagen (75). Lyons and Schwartz demonstrated some time ago that ascorbate had enormous effects on the secretion rate constant for collagen (12), and it is also well known that underhydroxylation of collagen leads to very high rates of intracellular degradation, presumably with accumulation of collagen in secretory compartments such as the endoplasmic reticulum and the Golgi. Similar accumulation can occur in certain forms of osteogenesis imperfecta, where reduced collagen mRNA has been associated with missense mutations. It is not clear how this secretory effect relates to the transcriptional activity of collagen genes, and we are unaware of any direct evidence of an ascorbate response element within either the collagen or elastin promoters. If further studies support the concept that the inverse effects of ascorbate

on collagen and elastin are mediated through lipoperoxides or other oxidative responses, this could have important implications for the control of accumulation of these two proteins during inflammatory events and subsequent tissue repair.

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