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Palmitoyl Ascorbate: Selective Augmentation of Procollagen mRNA Expression Compared With L-Ascorbate in Human Intestinal Smooth Muscle Cells

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Abstract The effect of 6-O-palmitoyl ascorbate on procollagen mRNA levels, collagen synthesis, and collagen secretion was investigated and compared with the effect of L-ascorbate in human intestinal smooth muscle (HISM) cells in vitro. Collagen synthesis, determined by the incorporation of ^{3}H-proline into pepsin-resistant, salt-precipitated collagen, increased in a concentration-dependent manner in response to palmitoyl ascorbate. There was a twofold increase in collagen synthesis at 2.5 and 5 μM. By contrast, L-ascorbate was required at 4–5 times the concentration for the same response. However, at 20 μM, both palmitoyl and L-ascorbate induced similar 2.7-fold increases in collagen synthesis. Palmitoyl ascorbate induced a 1.6- and 3.5-fold increase in steady-state levels of procollagen I and III mRNA levels respectively, whereas L-ascorbate had no effect. Palmitoyl ascorbate and L-ascorbate induced similar increases in the amounts of newly synthesized procollagen secreted into the medium and in the amounts of collagen types I, III and V accumulating in the cell layer. There was no effect of either palmitoyl ascorbate or L-ascorbate on the activity of a procollagen α_2 (I) promoter construct transiently transfected into HISM cells. Palmitoyl ascorbate augments HISM cell procollagen synthesis and mRNA levels more efficiently than L-ascorbate. This property may be due to the greater resistance of the ascorbate ester to oxidation and suggests that palmitoyl ascorbate could be an important agent for studies of collagen synthesis in vitro. J. Cell. Biochem. 73:312–320, 1999.

Key words: collagen; HISM cell; ascorbic acid; tissue repair; intestine; gene expression; collagen secretion; collagen synthesis

Ascorbic acid is a critical component for the biological expression, synthesis, and secretion of collagen. This essential vitamin acts as a cofactor in the hydroxylation of prolyl and lysyl residues in procollagen molecules by the enzyme prolylhydroxylase (lysylhydroxylase) [Jimenez et al., 1973; Berg and Prockop, 1973]. Ascorbic acid also increases steady-state levels of procollagen mRNA in some fibroblast lines [Geesin et al., 1988; Tajima and Pinnell, 1996], but not in human intestinal smooth muscle (HISM) cells [Graham et al., 1995b].

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A major problem in cell culture studies, and therapeutic applications, of ascorbic acid is the extreme instability of the compound resulting from its oxidation [Peterkofsky, 1972; Geesin et al., 1993; Austria et al., 1997]. In order to overcome this problem, attempts have been made to use various esters of ascorbic acid such as the fatty acid ester palmitoyl ascorbate and the phosphated, inorganic water-soluble salt of ascorbic acid, L-ascorbate phosphate.

Intestinal smooth muscle cells play a major role in the maintenance and repair of the intestinal wall. Defective extracellur matrix and inadequate repair in the intestine lead to spontaneous perforation, as seen in Ehlers Danlos type IV [Prockop and Kivirikko, 1984], and to morbid pathology such as fistula formation in Crohn's disease [Graham et al., 1992]. Studies

of HISM cells are in progress to determine the cellular and molecular factors that regulate collagen metabolism by these cells [Graham et al., 1995a, 1996]. Because ascorbic acid is so critical to this process of collagen expression, close attention has been paid to the requirements of these cells for ascorbate and to how the problem of ascorbate degradation can be minimized. In a previous study, the phosphated salt of L-ascorbate [Hata and Senoo, 1989] was found not to confer any advantage over ascorbic acid in HISM cells [Graham et al., 1995b]. Subsequent studies in human foreskin fibroblasts have demonstrated that the acylated ascorbate, 6-O-palmitoyl ascorbic acid, stimulated collagen synthesis, and at a concentration that was one-fifth the concentration of L-ascorbic acid necessary for the same effect [Rosenblat et al., 1998]. Because those data suggested that palmitoyl ascorbate could be a potentially useful, and more stable, derivative of ascorbic acid, experiments were performed to determine whether collagen synthesis by HISM cells was responsive to palmitoyl ascorbate. In addition, the effect of palmitoyl ascorbate on procollagen mRNA levels, procollagen secretion, and the transcriptional activity of a procollagen promoter were studied. The results demonstrate that palmitoyl ascorbate stimulated collagen synthesis in HISM cells, again at approximately one-fifth the concentration of L-ascorbic acid. In addition, palmitoyl ascorbate induced a threefold increase in procollagen mRNA levels–a potent response, considering that L-ascorbic acid had no effect. Interestingly, despite the greater effect of palmitoyl ascorbate on procollagen mRNA levels, L-ascorbate, and palmitoyl ascorbate had similar effects on the induction of procollagen secretion. Studies using a transiently transfected procollagen α_2 (I) promoter construct in HISM cells demonstrated that the augmentation of steady-state procollagen mRNA levels by palmitoyl ascorbate was not due to transcriptional activation of the procollagen I gene.

MATERIALS AND METHODS

Cell Culture

HISM cells were isolated from human jejunum by collagenase digestion as previously described [Graham et al., 1984]. Cells grown in primary culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MA) in a humidified atmosphere of 7% CO_2 were passaged for use in experiments. Cells were used from passages 2–6.

Preparation of Suspensions of 6-O-Palmitoyl and Linolenyl Ascorbate

For collagen synthesis experiments, 6-O-palmitoyl ascorbate and 6-O-linolenyl ascorbate suspensions were prepared by adding an ethanol solution of the acylated ascorbate to the media to achieve a final ethanol concentration up to 0.1–0.5%. At this concentration, ethanol did not affect collagen synthesis.

For the RNA experiments, 6-O-palmitoyl ascorbate suspension was prepared by either of two methods: (1) palmitoyl ascorbic acid in a final concentration of 1 mM was vigorously mixed with a Vortex with phosphate buffer (10 mM, pH 10.7) for 5 min, the pH adjusted to 6.8 with phosphoric acid [Rosenblat et al., 1998]; and (2) palmitoyl ascorbate or linolenyl ascorbate were solubilized in ethanol and then added to the media in a final ethanol concentration of 0.5–0.1%.

Collagen Synthesis

HISM cells trypsinized from culture dishes were seeded in 24-well microculture plates (Corning Glass Works, Corning, NY) at a density of 20,000 cells/well with 1 ml DMEM supplemented with 10% FBS. When cells were confluent in the wells (day 7 of culture), medium was removed and replaced with 1 ml of DMEM supplemented with 0.5% FCS; 24 h later, DMEM was additionally supplemented with a mixture containing 100 μg/ml β-aminopropionitrile, L-[5-^{3}H]proline (10 mCi/ml, specific activity 30 Ci/mmol, Amersham International, Buckinghamshire, England) and either L-ascorbic acid or 6-O-palmitoyl ascorbate (Sigma Chemical Co., St. Louis, MO) or 6-O-linolenyl ascorbic acid (Scotia Pharmaceutica, Stirling, Scotland). After a further 24 h, cells and media were harvested for determination of ^{3}H-proline incorporation into pepsin-resistant, salt-precipitated collagen [Webster and Harvey, 1997]. Briefly, extracellular ^{3}H-labeled collagen was extracted for 4 h at room temperature with 0.5 M acetic acid containing pepsin (Worthington Biochemical Corporation, Freehold, NJ) at a final concentration of 0.5 mg/ml. The extracted collagen was purified by successive salt precipitations at acid and neutral pH and then by precipitation in 20% ethanol. The final precipitate was solubi-

lized in 0.5 M acetic acid, placed in a scintillation liquid, and the radioactivity determined. Results were averaged from four identically treated wells. An aliquot of the trypsinized cells was counted in a hemocytometer.

Procollagen Secretion and Cell-Associated Collagen Types

Procollagen secretion and cell-associated collagen types were determined by the demonstration of newly synthesized, radiolabeled procollagen accumulating in media and in the cell layer by slab gel electrophoresis as previously described [Graham et al., 1995b]. HISM cells were grown to confluence in 100-mm culture dishes, in medium supplemented with 10% FBS. After 24 h in medium containing 0.5% serum, cells were washed twice with fresh medium and incubated for 6 or 24 h with medium containing L-[5-^{3}H]proline (10 μCi/ml, specific activity 30 Ci/mmol, Amersham International, Buckinghamshire, England), without, or with L-ascorbate (10 μM) or palmitoyl ascorbate (10 μM). The medium was then aspirated, the cells scraped off the plates, and the medium and cells incubated separately in 0.5 M acetic acid overnight at 4°C. Medium and cellular noncollagenous proteins were then pelleted by centrifugation (7,000*g*, 4°C). The supernantants were either neutralized by the addition of NaOH for the quantitation of procollagen in media or incubated with pepsin (Worthington Biochemical Corp.) overnight at 4°C for the determination of collagen types in the cell layer. Collagenous proteins were then precipitated in ethanol, air dried, extracted in Laemmli's buffer [Laemmli, 1970] and identical aliquots separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously published [Graham et al., 1995b]. For the demonstration of type III collagen bands, pepsin-digested cell layer samples were incubated for 1 h with dithiothreotol before the gel run as previously published [Graham et al., 1987, 1988]. Gels were enhanced in 1 M sodium salicylate, and exposed to photographic film.

RNA Extraction and Northern Analysis of Procollagen I and III mRNA

HISM cells were grown to confluence in 100-mm dishes and were then incubated under test conditions for 24 h according to the protocol described above. The cells were then harvested and total RNA isolated by extraction with Ultarspec RNA isolation system (Biotec Laboratories, Houston, TX). The RNA was separated by electrophoresis on a 1% agarose gel containing 5% formaldehyde and transferred to nitrocellulose membrane. The membrane was baked at 42°C for 2 h and was hybridized sequentially with cDNA probes to procollagen α_1 (I), and α_1 (III) [Graham et al., 1996] and then to 18S ribosomal mRNA after ^{32}P-labeling of the probes by random priming (Promega, Madison, WI). The hybridization signals were visualized by autoradiography and quantitated by scanning densitometry. Procollagen mRNA levels were expressed as a ratio of the procollagen to the 18S signals.

Determination of the Effect of Ascorbate and Its Derivatives on Transcriptional Activity of the Procollagen α_2 (I) Promoter Procollagen α_2 (I) Promoter Construct

A construct containing 3.5 kb of the procollagen α_2 (I) promoter fused to the chloramphenicol acetyltransferase (CAT) gene -pMS-3.5/CAT was kindly provided by Dr. Francesco Ramirez. The 3,500-base pair (bp) region located immediately upstream of the transcriptional start site of the human procollagen α_2 (I) gene contains all the sequences necessary for cell-specific transcription [Boast et al., 1990] and was derived from a 3.5-kb *Eco*R1/Sph1 subclone spanning positions −3500 to +58 on the procollagen α_2 (I) promoter [Dickson et al., 1985].

Transient Transfection Experiments

HISM cells at a density of 1×10^6 cells per 100-mm dish were incubated overnight in 10 ml DMEM supplemented with 10% FBS. A total of 15 μg of the pMS-3.5/CAT plasmid and 3 μg of the PGL2 luciferase control vector plasmid (Promega) as a standard were mixed in 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2 × HBS (50 mM Hepes, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4). The mixture was incubated for 30 min at room temperature; 1 ml was then added dropwise to the medium in the culture plates. After 5 h of incubation with the calcium phosphated DNA solution, the cells were shocked with 15% glycerol for 2 min. The transfection solution was then replaced by DMEM supplemented with 10% FBS for a further 16 h. Cells were then washed twice and incubated under test conditions according to the protocol described above. After 24 h exposure to the test conditions, the cells were washed three times

with phosphate-buffered saline (PBS) and lysed in 800 µl of 1× Reporter Lysis Buffer (Promega). Cell extracts containing the same amount of luciferase activity were incubated in a reaction mixture containing [^{14}C]-chloramphenicol and n-butyryl coenzyme A at 37°C for 4 h. CAT activity of cell extracts was determined by thin-layer chromatography (TLC) and autoradiography to demonstrate butyrylated chloramphenicol spots, which migrate faster than the unmodified chloramphenicol substrate.

Luciferase Assay

The luciferase activity of 100 µl lysate of transfected cells was quantitated in the presence of 360-µl reaction buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KPO_4, pH 7.8, 2 mM ATP, 1 mM dithiothreotol) and 200 µl Luciferin buffer (0.2 mM D-luciferin, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM dithiothreotol), using an LB9501 Luminometer (Berthold Systems, Aliquippa, PA).

RESULTS

Effect of L-Ascorbic Acid and Palmitoyl Ascorbate Acid on Collagen Synthesis by HISM Cells

Ascorbic acid induced a concentration-dependent increase in collagen synthesis in a range of concentration up to 20 mM (Fig. 1). Palmitoyl ascorbate induced a similar response, a 2.7-fold maximal effect at 20 mM. The response to palmitoyl ascorbate, however, was shifted to the left—a near-maximal twofold response was seen at 2.5 and 5 mM—one-fourth to one-fifth the concentration of L-ascorbic acid required for the same response. A similar stimulation of collagen synthesis was observed when HISM cells were treated with another fatty acid ester of ascorbate, linolenyl ascorbate (data not shown).

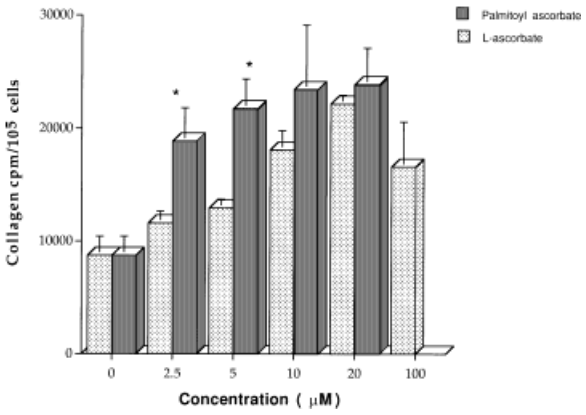

Fig. 1. Effect of palmitoyl ascorbate and L-ascorbate on collagen synthesis by human intestinal smooth muscle (HISM) cells. HISM cells, confluent in 24-well culture plates, were exposed to either palmitoyl or L-ascorbate for 24 h in the presence of medium supplemented with 0.5% fetal bovine serum (FBS). Collagen synthesis was determined by the incorporation of ^{3}H-proline into pepsin-resistant, salt-precipitated collagen [Webster and Harvey, 1997]. Each data point represents the mean and S.E.M. of radioactivity in four replicate wells normalized to 10^5 cells per well. The data are respresentative of three separate experiments. *, value for palmitoyl ascorbate significantly different from L-ascorbate, $P < 0.05$ by paired Student's *t*-test.

Effect of L-Ascorbic Acid and Palmitoyl Ascorbate on Type I and III Procollagen mRNA Levels in HISM Cells

Previous studies have demonstrated that in HISM cells, ascorbic acid affected procollagen mRNA levels only minimally, whereas in fibroblasts, ascorbic acid induced two- and threefold increases in procollagen I and III levels, respectively [Graham et al., 1995b]. It was therefore intriguing to determine whether the greater sensitivity of HISM cell collagen synthesis to palmitoyl ascorbate was associated with an increase in procollagen mRNA. When ascorbate-deprived HISM cells were exposed for 24 h, L-ascorbic acid (10–100 µM) had no effect on either type I or type III procollagen mRNA levels (Fig. 2A,B). On exposure to palmitoyl ascorbate, there was a concentration-dependent increase in procollagen mRNA levels that was maximal at 10 µM. Procollagen I mRNA levels were increased 1.6-fold when the palmitoyl ascorbate (10 µM) was dissolved in ethanol, and 1.4-fold when suspended in buffer (Fig. 2A). For comparison, transforming growth factor-β1 (TGF-β1) (10 pM) induced a 2.6-fold increase in procollagen mRNA (Fig. 2A). When the effect on procollagen III mRNA levels was examined, palmitoyl ascorbate induced a 3.5-fold increase (Fig. 2B). This greater sensitivity of type III procollagen mRNA to modulation was described previously in HISM cells [Graham et al., 1996]. Linolenyl ascorbate, another fatty acid ester of ascorbic acid, induced a much smaller—30%—increase in procollagen III mRNA (Fig. 2B).

Effect of L-Ascorbic Acid and palmitoyl Ascorbate on Secretion of Procollagen and Synthesis of Collagen Types I, III, and V

One of the critical components of the effect of ascorbate on procollagen metabolism is as a cofactor in the enzymatic hydroxylation of proline residues by prolyl hydroxylase. This step is

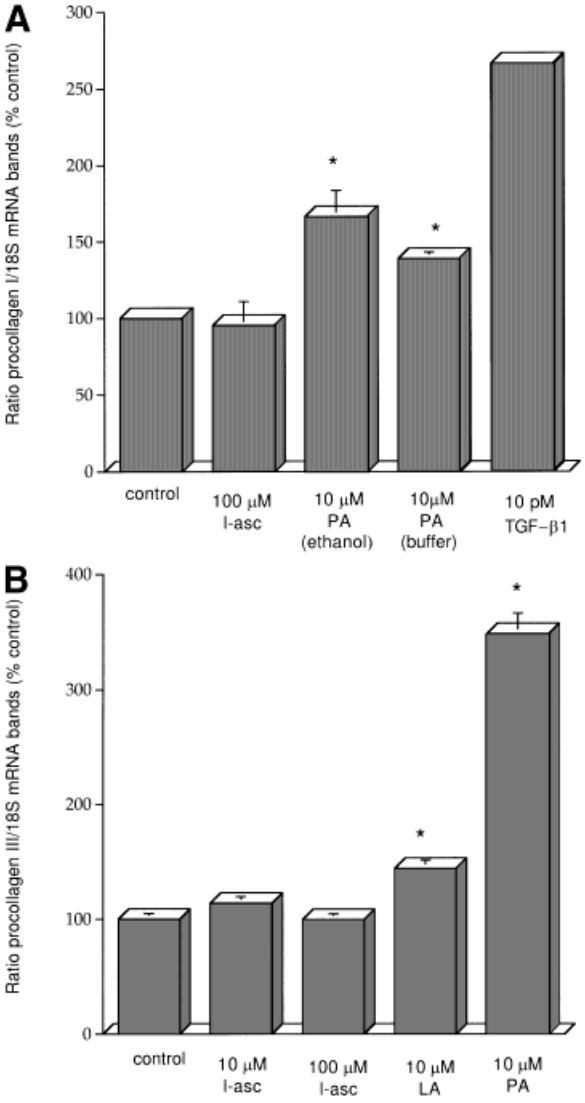

Fig. 2. Effect of palmitoyl ascorbate and L-ascorbate on procollagen I **(A)** and procollagen III **(B)** mRNA levels in human intestinal smooth muscle cells. HISM cells, confluent in 100-mm culture dishes were exposed to either palmitoyl- or L-ascorbate for 24 h in the presence of medium supplemented with 0.5% fetal bovine serum. Cells were then harvested, RNA isolated, and procollagen mRNA levels determined by Northern blot analysis after sequential hybridization with procollagen I and III, and then 18S ribosomal cDNA probes. Procollagen and 18S mRNA bands were quantitated by scanning densitometry. Each data point represents the mean and S.E.M. of the ratio of procollagen to 18S bands from three separate culture plates. L-asc, L-ascorbate; PA, palmitoyl ascorbate; LA, linolenyl ascorbate. ethanol/buffer, palmitoyl ascorbate dissolved in either ethanol or buffer. *, significantly greater than the values for L-ascorbate and control, $P < 0.05$ by paired Student's *t*-test.

necessary for folding of the procollagen triple helix and is essential for procollagen secretion [Blanck and Peterkofsky, 1975]. It was therefore intriguing to compare the effects of ascorbate and palmitoyl ascorbate on the secretion of procollagen. Interestingly, L-ascorbate and palmitoyl ascorbate at 10 μM induced similar increases in the amount of radiolabeled, newly synthesized procollagen into the medium (Fig. 3A). Both vitamins also induced marked increases in the amount of fibronectin secreted (Fig. 3A).

When the phenotypes of the newly synthesized radiolabeled collagen in the cell layer were examined, type I collagen, the major collagen in the cell layer, was increased similarly by L-ascorbate and palmitoyl ascorbate. In addition, L-ascorbate and palmitoyl ascorbate induced similar, marked increases in the intensity of the type III and type V collagen bands (Fig. 3B).

Effect of L-Ascorbate and Palmitoyl Ascorbate on Fibroblast Collagen Synthesis and mRNA Levels

Because there are major differences in the way HISM cells and fibroblasts respond to L-ascorbate [Graham et al., 1995b], it was important to determine whether there were also similar differences in the response to palmitoyl ascorbate. In the line of human dermal fibroblasts tested, palmitoyl ascorbate and ascorbic acid at 10 μM induced similar increases–threefold–in collagen synthesis. TGF-β_1 (40 pM), for comparison, induced a sevenfold increase in collagen synthesis in the presence of L-ascorbate (Fig. 4). Interestingly, in fibroblasts, these increases in collagen synthesis were mirrored by similar increases in procollagen III mRNA—twofold by both palmitoyl ascorbate and L-ascorbic acid. TGF-β_1, for comparison, induced a fourfold increase (Fig. 5). In fibroblasts, therefore, procollagen mRNA is augmented by both palmitoyl- and L-ascorbate, whereas in HISM cells, procollagen mRNA is increased by palmitoyl-ascorbate, and not by L-ascorbate.

Effect of L-Ascorbic Acid and Palmitoyl Ascorbate on Transcriptional Activity of the Procollagen α_2 (I) Promoter

One possible mechanism for the greater effect of palmitoyl ascorbate over L-ascorbic acid on procollagen mRNA levels in HISM cells is by the induction of transcriptional activity of the procollagen gene. In order to examine this question, HISM cells were transiently transfected with a procollagen α_2 (I) promoter construct plasmid containing a CAT reporter gene and

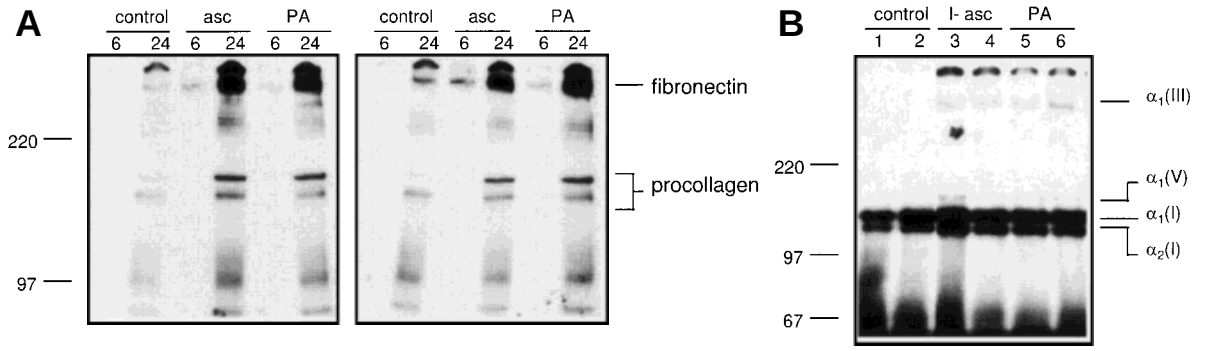

Fig. 3. Effect of palmitoyl ascorbate and L-ascorbate on procollagen secretion **(A)** and cell-associated collagen types **(B)**. Confluent human intestinal smooth muscle (HISM) cells were exposed to either palmitoyl ascorbate or L-ascorbate (10 mM) for 24 h and then incubated with L-[5-^{3}H]proline. Medium and cells were harvested and collagenous proteins solubilized in 0.5 N acetic acid. Supernatants were then either neutralized (procollagen in media, A) or pepsin-digested (collagen types in cell layer, B). Equal aliquots of supernatants were loaded into each lane and procollagen and collagen bands demonstrated by polyacrylamide slab gel electrophoresis. The two panels in A represent media proteins from two of three separate sets of culture plates. The two lanes in each condition in B are replicates for that condition. Procollagen and fibronectin bands (A) are identified as previously published [Graham et al., 1995b]. α_1 (III) collagen bands were demonstrated by delayed reduction, and α chains of collagen types I, III, V identified (B), as previously published (Graham et al., 1987, 1988). asc, L-ascorbate; PA, palmitoyl ascorbate; 6, 24, medium harvested at 6 and 24 h.

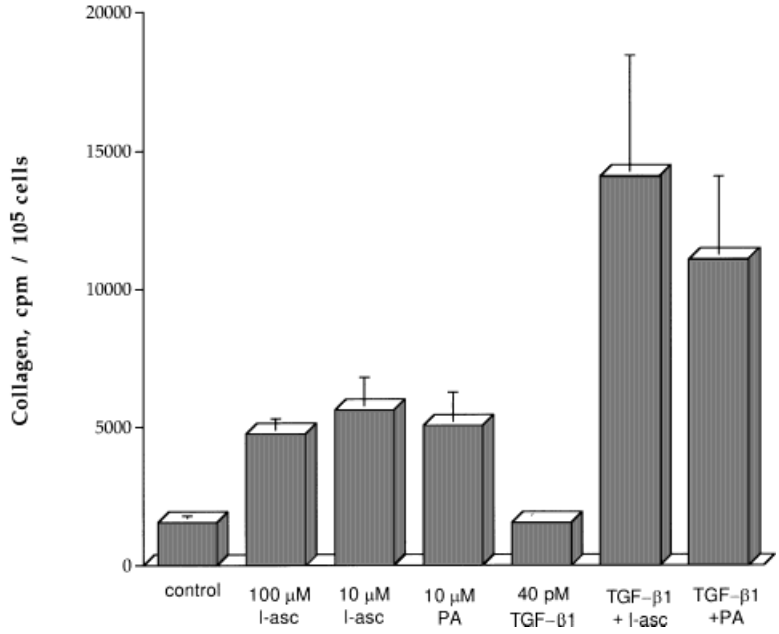

Fig. 4. Effect of palmitoyl ascorbate and L-ascorbate on collagen synthesis by human dermal fibroblasts. Fibroblasts, confluent in 24-well culture plates, were exposed to either palmitoyl or L-ascorbate for 24 h in the presence of medium supplemented with 0.5% fetal bovine serum (FBS). Collagen synthesis was determined by the incorporation of ^{3}H-proline into pepsin-resistant, salt-precipitated collagen [Webster and Harvey, 1997]. Each data point represents the mean and S.E.M. of radioactivity in four replicate wells normalized to 10^5 cells per well. AA, L-ascorbate; PA, palmitoyl ascorbate.

exposed to either L-ascorbic acid or palmitoyl ascorbate. Transcriptional activity of the promoter was determined by CAT assay. Neither palmitoyl- nor L-ascorbate induced any increase in the activity of the transfected promoter construct. By contrast, TGF-β (10 pM) induced a marked increase in CAT activity, as compared with control cells (Fig. 6). Identical findings were seen in three separate experiments.

DISCUSSION

The hydrophobic derivative of L-ascorbic acid, 6-O-palmitoyl ascorbate, was developed for use as an agent with increased antitumor activity compared with L-ascorbic acid [Miwa and Yamazaki, 1986]. Palmitoyl ascorbate was also found to be active in a number of other biochemical and physiological processes, including DNA synthesis [Kageyama et al., 1996], phospho-

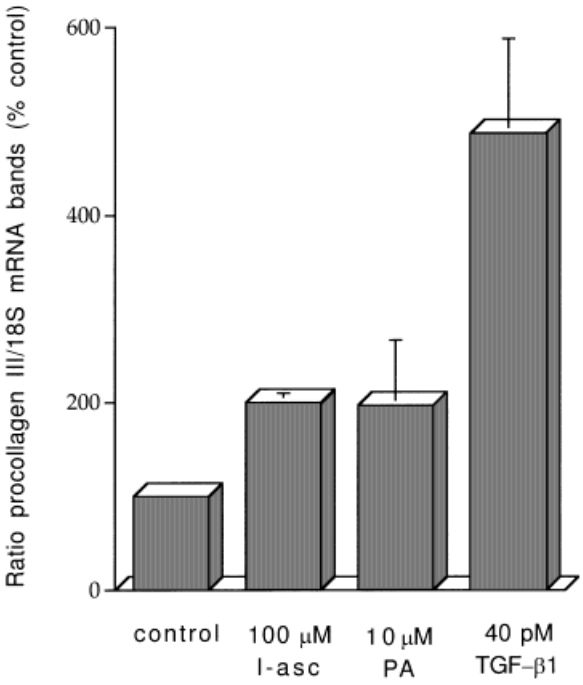

Fig. 5. Effect of palmitoyl ascorbate and L-ascorbate on procollagen III mRNA levels in human dermal fibroblasts. Fibroblasts, confluent in 100-mm culture dishes were exposed to either palmitoyl- or L-ascorbate for 24 h in the presence of medium supplemented with 0.5% fetal bovine serum (FBS). Cells were then harvested, RNA isolated, and procollagen mRNA levels determined by Northern blot analysis after sequential hybridization with procollagen III, and then 18S ribosomal cDNA probes. Procollagen and 18S mRNA bands were quantitated by scanning densitometry. Each data point represents the mean and S.E.M. of the ratio of procollagen to 18S bands from three separate culture plates.

lipid metabolism [Kageyama et al., 1991], and post-transcriptional induction of ornithine decarboxylase [Matsui-Yuasa et al., 1989]. Additionally, palmitoyl ascorbate has been shown to inhibit lipid peroxide-induced damage and several oxidative functions in human neutrophils [May et al., 1996]. Another hydrophobic derivative of L-ascorbic acid, linolenyl ascorbate, was recently devloped as a more stable delivery system for linolenic acid.

Recent studies have demonstrated that 6-O-palmitoyl ascorbate (but not palmitic acid or its combination with ascorbate) stimulated collagen synthesis in cultured human foreskin fibroblast at lower concentrations than L-ascorbic acid [Rosenblat et al., 1998]. In the current studies, we have demonstrated that the effect of palmitoyl ascorbate on collagen synthesis in HISM cells is similar to that previously seen in fibroblasts, namely, a similar augmentation, but at lower concentrations than L-ascorbic acid. In addition, the current studies have demonstrated that palmitoyl ascorbate significantly increased levels of procollagen I and III mRNA in HISM cells whereas L-ascorbic acid had no discernable effect.

Linolenyl ascorbate had effects similar to those observed with palmitoyl ascorbate with regard to collagen synthesis. However, the effect on mRNA levels was significantly less. This is probably explained by the different biochemical properties of linolenic acid, a polyunsaturated fatty acid, which has been reported to interfere with arachidonic acid metabolites and collagen metabolism [Beno et al., 1993; Bienkowski and Gotkin, 1995].

Discordance between procollagen mRNA levels and collagen synthesis rates have been previously described in certain cell lines [Phillips et al., 1992]. Rowe and Schwartz [1983] demonstrated that α,α-dipyridyl, an inhibitor of hydroxylases, caused a two- to threefold decrease in the rate of procollagen synthesis without affecting the level of procollagen mRNA in pri-

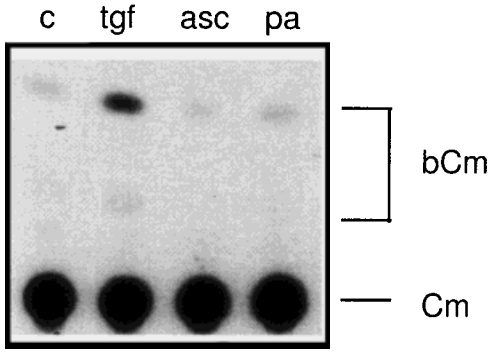

Fig. 6. Effect of palmitoyl ascorbate and L-ascorbate on transcriptional activity of the collagen α_2 (I) promoter. Human intestinal smooth muscle (HISM) cells were transfected with the pMS-3.5/CAT plasmid (a construct containing 3.5 kb of the procollagen α_2 (I) promoter fused to the chloramphenicol acetyltransferase (CAT) gene)(Dickson et al., 1985), and the PGL2 luciferase control vector plasmid, and then exposed to palmitoyl ascorbate or L-ascorbate (10 μM) for 24 h. Cell extracts containing the same amount of luciferase activity were incubated in a reaction mixture containing [^{14}C]-chloramphenicol and n-butyryl coenzyme A. CAT activity was determined by thin-layer chromatography and autoradiography to demonstrate the two butyrylated products of chloramphenicol (bCm) which migrate faster than the unmodified chloramphenicol substrate (Cm). The CAT activity of cells exposed to palmitoyl ascorbate (pa) or L-ascorbate (asc) was no different from control (c), whereas TGF-β (10 pM, tgf), a positive control, induced a marked increase. Identical findings were seen in three separate experiments.

mary avian tendon cells. Similar observations were also made by Kurata et al. [1993] and Tolstoshev et al. [1981]. Hitomi and Tsukagoshi [1996] suggested that, in reponse to L-ascorbic acid, there might not be direct correspondence between the level of procollagen mRNA and the rate of procollagen synthesis. Our observations would suggest that the same applies to the response to palmitoyl ascorbate in HISM cells.

The current studies have demonstrated that 6-O-palmitoyl ascorbate has a more potent effect than L-ascorbic acid on collagen synthesis in HISM cells. Two important questions need to be addressed: (1) what is the reason for the greater sensitivity to 6-O-palmitoyl ascorbate? and (2) why is there discordance between effects on mRNA levels and collagen synthesis for L-ascorbic acid, but not for 6-O-palmitoyl ascorbate?

One explanation for the greater potency of palmitoyl ascorbate is that it has increased resistance to enzymatic and auto-oxidation. This hypothesis has been suggested previously [Rosenblat et al., 1998], and the increased stability of the ascorbate ester has been documented [Austria et al., 1997]. This property of stability is very pertinent in the cell culture environment where the half life of intracellular ascorbate is short—only 2 h [Kipp and Schwarz, 1990]. A more stable form of ascorbate added to culture medium would certainly lead to more sustained intracellular levels and therefore greater sensitivity to a given concentration in the medium.

A similar phenomenon could account for the lack of discernable effect of L-ascorbic acid on procollagen mRNA levels. High intracellular levels of L-ascorbic acid might be present within the first hours of exposure and could initiate the transcriptional response for increased mRNA levels and subsequent translational machinery for increased protein synthesis. The lack of sustained L-ascorbate levels due to degradation could then lead to a decrease in mRNA levels to baseline by the time RNA was harvested at 24 h. By contrast, palmitoyl ascorbate, because of its greater stability, may induce a more sustained increase in steady-state mRNA levels in HISM cells. The augmentation of procollagen mRNA levels by both L- and palmitoyl-ascorbate in fibroblasts, suggests differences in the metabolism of ascorbate between the two cell lines. These hypotheses will require more detailed kinetic studies for substantiation.

The transient transfection studies in the current studies demonstrate that palmitoyl ascorbate did not induce transcriptional activity of the procollagen promoter, whereas TGF-β did. These findings suggest that palmitoyl ascorbate does not increase procollagen mRNA levels by activating procollagen gene expression, at least in the same manner as TGF-β. It is also possible that this response is induced by stabilising procollagen mRNA, as previously demonstrated for ascorbate in primary avian tendon cells [Lyons and Schwartz, 1984]. This possibility will also need to be tested in future work.

These studies have demonstrated that 6-O-palmitoyl ascorbate could be an important replacement for ascorbic acid in investigations of collagen expression that use human smooth muscle cells and fibroblasts and that it could also be a more stable form of ascorbic acid for use in therapeutic applications in vivo.

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