d-α-Tocopherol Inhibits Collagen α_1(I) Gene Expression in Cultured Human Fibroblasts

Modulation of Constitutive Collagen Gene Expression by Lipid Peroxidation

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Abstract

Ascorbic acid stimulates collagen gene transcription in cultured fibroblasts, and this effect is mediated through the induction of lipid peroxidation by ascorbic acid. Quiescent cultured fibroblasts in the absence of ascorbic acid have a high constitutive level of collagen production, but the mechanisms of collagen gene regulation in this unstimulated state are not known. Because lipid peroxidation also occurs in normal cells, we wondered if lipid peroxidation plays a role in the regulation of basal collagen gene expression. Inhibition of lipid peroxidation in cultured human fibroblasts with d-α-tocopherol or methylene blue decreased the synthesis of collagen, the steady-state levels of procollagen α_1(I) mRNA and the transcription of the procollagen α_1(I) gene. This effect on collagen gene expression was selective and not associated with cellular toxicity. Thus, these experiments suggest a role for lipid peroxidation in the modulation of constitutive collagen gene expression. (*J. Clin. Invest.* 1991. 87:2230–2235.) Key words: collagen gene • aldehydes • antioxidants • methylene blue • oxidative stress

Introduction

Increased deposition of collagen in many pathological disorders, including atherosclerosis (1), hepatic cirrhosis (2, 3), and interstitial lung disease (4), is detrimental to normal organ function. Because fibroblasts have a high constitutive level of collagen gene expression both in vitro (5–8), and in vivo (9), cultured fibroblasts have been used as a model to study the mechanisms regulating collagen synthesis (5–8, 10–15). Four factors have been identified to date which stimulate collagen gene transcription in cultured fibroblasts: ascorbic acid (10, 11), a fibrogenic factor (12), acetaldehyde (13), and transforming growth factor β under some conditions (14) but not others (15). However, the mechanisms responsible for the high constitutive expression of collagen genes in unstimulated cultured fibroblasts remain unknown.

We have recently suggested that lipid peroxidation mediates the stimulation of collagen gene transcription by ascorbic acid (11). In addition, the fibrogenic factor purified from the liver of rats treated with thioacetamide or CCl_4 (12, 16) may be the result of lipid peroxidation induced by these hepatotoxins. Because lipid peroxidation also occurs in unstimulated quiescent cultured fibroblasts (11, 17, 18), as well as in normal tissues in vivo (Houglum, K., unpublished observations; 19–22), we wondered if products of lipid peroxidation might modulate basal collagen gene expression.

In this study, we show that inhibition of basal lipid peroxidation with d-α-tocopherol and methylene blue results in a decrease of the constitutive collagen α_1 (I) gene expression in normal cultured fibroblasts.

Methods

Materials. Biodyne transfer membrane was purchased from Pall (Glen Cove, NY). Sources of other chemicals were: L-[5-^{3}H]proline (22 mCi/mmol) and aqueous counting scintillant fluid from Amersham Corp. (Arlington Heights, IL); chromatographically purified bacterial collagenase form III, from Advance Biofactures Co. (Lynbrook, NY); [α-^{32}P]2′-deoxycytidine 5′-triphosphate from ICN Biochemicals, Inc. (Irvine, CA); deoxynucleotides, and Klenow fragment of DNA polymerase I, from Pharmacia LKB Biotechnology (Piscataway, NJ); agarose and restriction endonucleases from BRL (Gaithersburg, MD); AG-50W-X8 and low melting agarose from Bio-Rad Laboratories, Inc. (Richmond, CA); CsCl from IBI (New Haven, CT); guanidine isothiocyanate from BRL (Gaithersburg, MD); thiobarbituric acid, d-α-tocopherol and propyl gallate from Sigma Chemical Co. (St. Louis, MO); Sephadex G-50 prespun columns from Boehringer Mannheim Biochemicals, Inc. (Indianapolis, IN); human fetal AF_2 fibroblasts, and the media used for their in vitro culturing from the Core Cell Culture Facility (University of California, San Diego, CA). Probucol was a generous gift of Merrell Dow Pharmaceuticals Inc. (Cincinnati, OH), and BW755c was a generous gift of Wellcome Research Laboratories (Kent, UK).

Fibroblast cultures. Human fetal AF_2 fibroblasts were used at subcultivations 5–21 as previously described (23). Cells were cultured under an atmosphere of 5% CO_2, 95% air in tissue culture dishes using MEM containing 10% FCS. Cells were plated at a density of 9×10^5/P-10 dish and radiolabeling studies were performed after 6 d as described below.

Measurement of collagen production. Confluent cell cultures were incubated in 10 ml of MEM with 10% FCS containing 0.1 mM L-proline, at 37°C in 5% CO_2, 95% air. The influence of d-α-tocopherol (10–50 μM), BW755c (100 μM), Probucol (15 μM), propyl gallate (10 μM), and methylene blue (10 μM), a scavenger of reducing equivalents (24), on quiescent cells was assessed by the addition of these compounds during the incubation period (20 h). After the incubation period, 0.2 mM ascorbic acid (a cofactor for prolyl and lysyl hydroxylases) and 20 μCi of L-[5^{3}H]proline were added and the incubation was continued for up to 4 h (23). Labeling of cells was terminated by cooling the plates to 0°C. Collagen and noncollagen protein production in combined cell layer and medium were determined by the collagenase method (25), as previously described (26–28). The radioactivity of colla-

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genase-sensitive and collagenase-insensitive proteins was used to calculate the relative rate of collagen production.

Evaluation of cellular toxicity. Loss of cellular lactic dehydrogenase was measured in the media of cell cultures as described previously (27). Cell counting was performed as described previously (27).

Determination of lipid peroxidation. Lipid peroxidation was determined by measuring thiobarbituric acid-reacting substances (TBARS)[1] in the cell layer as described by Ohkawa and co-workers (29), except that cells from two plates were combined and lysed in 0.12 M NaCl, 10 mM $NaPO_4$ and 50 μM butylated hydroxytoluene (BHT) by freeze-thaw. All control plates received 50 μM *d*-α-tocopherol or 10 μM methylene blue at the time of cell harvest. TBARS were measured fluorometrically (excitation, 515 nm; emission, 553 nm) using malondialdehyde (MDA) and tetramethoxypropane standards. The thiobarbituric acid–malondialdehyde complex was also determined by HPLC (30). This complex was eluted from a C18 column with 11% methanol (1 ml/min) after 5 min and measured at 546 nm.

Preparation of cDNA probes. The plasmid pHF677 (31) containing cDNA for the human α_1 (I) collagen was provided by Dr. F. Ramirez. The plasmid pKαI containing the cDNA from human α-tubulin was provided by Dr. N. Cowan (32). The plasmid pkb3.uw containing the human c-*fos* cDNA (33) was provided by Dr. I. Verma. Using the random primer synthesis method (34), the cDNA fragments were radiolabeled with α^{32}P-dCTP to sp act $\sim 1 \times 10^9$ cpm/μg DNA.

Northern blotting and slot blotting. AF_2 cells were incubated as described above with *d*-α-tocopherol (50 μM) and methylene blue (10 μM). Cells were processed as previously described (13), and RNA was isolated by the guanidine thiocyanate/phenol/chloroform extraction method (35). Total RNA was used for northern blotting as previously described (13, 36). The northern blot autoradiograms were quantitated by a scanning laser densitometer interfaced with an integrator.

Run-off transcription. The nuclear run-off transcription assay which was developed by Groudine et al. (37) and adapted by Wang and Calame (38) was used as described previously (13). The average experiment produced 1–2 cpm of radiolabeled RNA per nucleus.

Statistical analysis. All the results are expressed as mean±SEM unless stated otherwise. The Student's *t* test was used to evaluate the differences of the means between groups, accepting $P < 0.05$ as significant (39).

Results

Inhibition of lipid peroxidation by d-α-tocopherol. We first assessed whether lipid peroxidation occurred in quiescent cultured human fibroblasts. Cells grown under normal tissue culture conditions were found to have measurable TBARS, an index of lipid peroxidation (29, 30, 40). This was determined by the thiobarbituric acid assay using flurometric and HPLC detection of the malondialdehyde-thiobarbituric acid complex. Spurious lipid peroxidation induced during sample preparation was prevented by adding BHT to the lysis buffer (40). Additionally, BHT prevents propagation reactions which can occur in the acid milieu of the reaction mixture, thus malondialdehyde and lipid peroxides are predominantly measured under these conditions (40). A significant dose response reduction in TBARS (Fig. 1) was demonstrated when cell cultures were incubated with *d*-α-tocopherol for 20 h (control, 233±13 pm/mg protein). To insure that the observed effect of decreased TBARS was from the incubation of the cells with *d*-α-tocopherol, all control plates received the same amount of *d*-α-tocopherol at the time of harvest. Cellular toxicity due to *d*-α-tocopherol was not evident during the incubation as measured by the secretion of LDH into the culture media (311±5 vs. 330±30 IU, NS) and by cell counting ($3.0\pm0.1 \times 10^6$ vs. $3.2\pm0.4 \times 10^6$ cells per plate, NS).

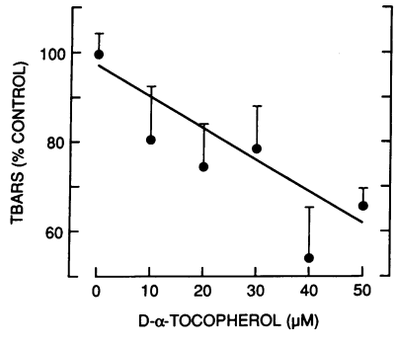

Figure 1. Dose-response inhibition of basal lipid peroxidation by *d*-α-tocopherol. Confluent cell cultures were incubated for 20 h in MEM and 10% FCS in the presence of *d*-α-tocopherol. TBARs were determined in cell layers as described in Methods. Percentage±SEM of control value (n = 10) for 10–40 μM (n = 2) and 50 μM (n = 10) *d*-α-tocopherol are shown with the regression line. $P < 0.001$ for both the regression line and 50 μM *d*-α-tocopherol.

Methylene blue is an oxidant (41), and will scavenge intracellular reducing equivalents, such as NADH, NADPH, and GSH (24, 42). When methylene blue was added to cultured fibroblasts, a decrease in TBARS similar to that noted for *d*-α-tocopherol (50 μM) (Fig. 1) was shown (65±4% of control). Because methylene blue interfered slightly (20%) with the fluorometric detection, methylene blue was added to the MDA standards used for TBARS determination, in an amount equal to that found in cell lysates. The mechanism by which methylene blue decreased TBARS in cell cultures under these experimental conditions is not known. A reduction of GSH might be expected to place cells at an increased risk for lipid peroxidation, however, this was not shown in other studies using red blood cells (41).

Inhibition of collagen gene expression by d-α-tocopherol and methylene blue. Cultured human fibroblasts were incubated in MEM, 10% FCS in the presence or absence of *d*-α-tocopherol for 20 h. Cells were then labeled for an additional 4-h period with [^{3}H]proline in the presence of ascorbic acid, a cofactor for prolyl and lysyl hydroxylases. The net production of collagen was decreased by *d*-α-tocopherol in a dose-dependent manner, whereas noncollagen protein production decreased slightly and independent of the dose (Fig. 2 and Table I). Similarly, BW 755c and Probucol, both antioxidants, inhibited basal collagen production without inducing cellular toxicity (Table II). In addition, the antioxidant propyl gallate (10 μM) (43) was found to decrease constitutive collagen production (data not shown). Methylene blue, a scavenger of reducing equivalents (24, 41), decreased TBARS in cell cultures and also inhibited basal collagen synthesis without affecting noncollagen proteins (Table I).

The step at which *d*-α-tocopherol acts to decrease collagen synthesis was studied. Total RNA was extracted from human fibroblasts incubated in the presence of *d*-α-tocopherol for 20 h. The fibroblast RNA was analyzed in northern blots, using human α-tubulin and procollagen α_1 (I) DNA probes. The latter was selected because collagen type I is the major collagen expressed in these cultured cells (36). The northern blots revealed that the RNAs were intact and that *d*-α-tocopherol does not alter the size of procollagen α_1 (I) and α-tubulin transcripts (Fig. 3). The two transcripts of the procollagen α_1 (I) (4.8 and

1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances.

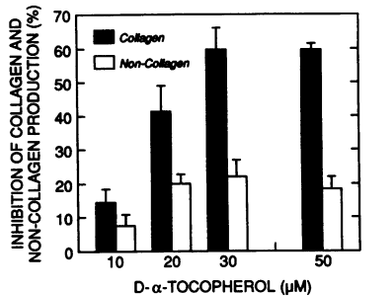

Figure 2. Dose-response effect of *d*-α-tocopherol on collagen production by human fibroblasts. Cell cultures were incubated with or without (control) *d*-α-tocopherol for 20 h and then labeled for 4 h with 10 μCi of [5-^{3}H]proline (0.1 mM) in the presence of 0.2 mM ascorbic acid. The relative rate of collagen and noncollagen production were calculated from the ^{3}H radioactivity in collagenase-sensitive and -resistant proteins, respectively. Percentage inhibition from control values for 10 μM (n = 9), 20 μM (n = 6), 30 μM (n = 9), and 50 μM (n = 4) *d*-α-tocopherol are shown. An equal number of control samples were used at each concentration. $P < 0.05$ at 20-, 30-, and 50-μM concentrations for collagen protein.

5.8 kb in length) have previously been described and represent alternative choices of polyadenylation sites (28). We found a marked decrease in procollagen α_1 (I) mRNA, with a moderate increase (in some but not all experiments) in α-tubulin mRNA, in cells incubated with *d*-α-tocopherol (Fig. 3). Similarly, methylene blue decreased procollagen α_1 (I) mRNA levels in cultured fibroblasts (Fig. 3).

Table I. Inhibition of Constitutive Collagen Production by d-α-Tocopherol and Methylene Blue

Experimental condition*	Collagen‡	Noncollagen protein‡	Relative rate of collagen production§
	dpm × 10^{-3}		%
Experiment 1			
Control	16.1±0.7	124.8±11.2	100±6
d-α-Tocopherol (50 μM)	6.6±2	102.3±4.4	52±2
Experiment 2			
Control	3.2±2	112.4±5.3	100±3
d-α-Tocopherol (30 μM)	1.2±0.1	91.6±1.0	46±2
Experiment 3			
Control	3.1±0.2	101.5±0.1	100±3
d-α-Tocopherol (10 μM)	2.6±0.1	93.0±11.1	92±6
d-α-Tocopherol (20 μM)	2.2±0.2	82.3±3.4	88±12
Experiment 4			
Control	8.0±0.3	120.5±2.3	100±4
Methylene blue (10 μM)	4.6±0.6	100.7±2.8	71±9
Experiment 5			
Control	7.0±0.4	79.2±5.8	100±12
Methylene blue (10 μM)	1.6±0.3	65.6±4.6	30±8

* Confluent cell cultures were incubated for 20 h in MEM, 10% FCS and then labeled in the presence of 0.2 mM ascorbic acid for 4 h at 37°C with 740 kBq of L-[5-^{3}H]proline. Experiments 1–5: No additions (control) or with the addition of *d*-α-tocopherol or methylene blue as indicated. ‡ Determined from the ^{3}H radioactivity incorporated into collagenase-sensitive and -resistant proteins after labeling cell cultures with 740 kBq of [5-^{3}H]proline for 4 h. Values are mean±SE of at least triplicate samples. $P < 0.05$ for collagen (*d*-α-tocopherol [experiments 1 and 2; experiment 3, 20 μM *d*-α-tocopherol] and methylene blue [experiments 4 and 5)]. § Calculated from the formula [^{3}H] collagen dpm/([^{3}H]noncollagen dpm × 5.4 + [^{3}H]collagen dpm), and expressed as percentage of control values. The SE reported is the SE of the individual samples in each group.

Table II. Inhibition of Constitutive Collagen Production by Probucol and BW-755C

Experimental condition*	Collagen‡	Noncollagen protein‡	Relative rate of collagen production§
	dpm × 10^{-3}		%
Experiment 1			
Control	3.3±0.2	52.7±3.1	100±8
BW-755c	2.2±0.3	56.9±2.4	62±6
Experiment 2			
Control	4.0±0.2	92.9±4.0	100±4
BW-755c	1.9±0.1	74.3±6.6	62±8
Experiment 3			
Control	2.8±0.2	88.0±7.4	100±7
Probucol	1.5±0.1	74.9±5.0	65±4

* Confluent cell cultures were incubated in MEM, and labeled in the presence of 0.2 mM ascorbic acid as described in Table I. Experiments 1–3: With no additions (control) or with the addition of BW-755c (100 μM) or Probucol (15 μM). ‡ Determined as described in Table I. Values are means±SE of triplicate samples; $P < 0.05$ for collagen (experiments 1–3). § Calculated as described in Table I.

To study the effect of a 20-h incubation with *d*-α-tocopherol on the transcriptional rates of both collagen and noncollagen protein genes in cultured fibroblasts, run-off transcriptional assays were utilized. Results of transcriptional run-off assays using nuclei isolated from untreated and *d*-α-tocopherol-treated fibroblasts are shown in Fig. 4. Procollagen α_1 (I) transcriptional activity was decreased ~ 50% by *d*-α-tocopherol. No effect on α-tubulin and a slight increase in c-*fos* gene transcription was noted.

Discussion

We have previously suggested that lipid peroxidation plays a role in the ascorbic acid–induced stimulation of collagen gene transcription (11). In this study we present evidence strongly

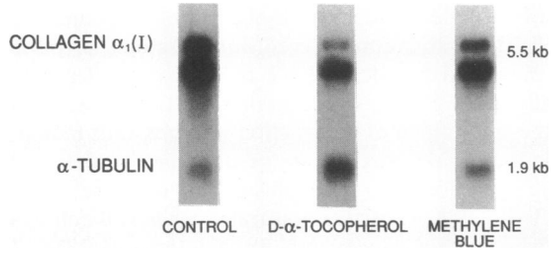

Figure 3. Northern blot analysis of human fibroblast collagen mRNA. Confluent human fibroblasts were incubated for 20 h in MEM and 10% FCS without additions (control) (lane *1*), or with *d*-α-tocopherol (50 μM) (lane *2*), or methylene blue (10 μM) (lane *3*). 10 μg of total RNA were electrophoresed on a formaldehyde 1% agarose gel and transferred to a nylon filter by capillary blotting. Filters were hybridized to radiolabeled human collagen α_1(I) and α-tubulin cDNA. This is a representative experiment of at least four independent experiments with *d*-α-tocopherol.

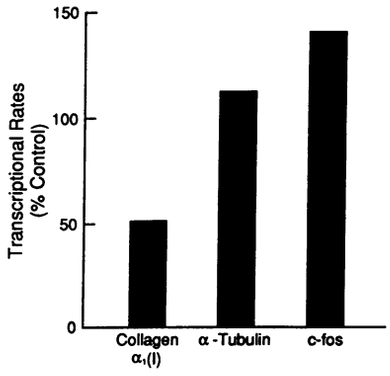

Figure 4. *d*-α-Tocopherol inhibits collagen α_1(I) gene transcription. Nuclei were isolated from control and *d*-α-tocopherol (50 μM) (*solid bars*)-treated human fibroblasts incubated as described for Fig. 3. Purified radiolabeled nascent RNAs (2×10^7 cpm) from each sample were hybridized to 1 μg each of plasmid DNA alone (background control) or containing the cDNAs collagen α_1(I), α-tubulin, and c-*fos* immobilized on nitrocellulose filters. The autoradiograms were quantitated by scanning laser densitometry and the data from two independent experiments combined.

supporting the hypothesis that lipid peroxidation, in the absence of ascorbic acid, modulates constitutive collagen α_1 (I) gene expression in normal cultured fibroblasts. Inhibition of basal lipid peroxidation with *d*-α-tocopherol, a lipophilic antioxidant, is associated with a decrease in constitutive collagen synthesis, collagen α_1 (I) mRNA steady-state levels, and transcription of this gene. Similarly, methylene blue, a scavenger of reducing equivalents (24, 41), inhibited basal lipid peroxidation and decreased basal collagen synthesis and collagen α_1 (I) mRNA steady-state levels. In addition, we have shown that BW 755c and Probucol, both antioxidants, also inhibit collagen production.

The mechanisms by which basal lipid peroxidation modulates the transcription of the collagen α_1 (I) gene are unknown. Reactive aldehydes are formed as a result of the oxidative breakdown of polyunsaturated fatty acids, and these aldehydes may form covalent links with various amino acid residues of proteins (44, 45). The function of various proteins are altered by the formation of aldehyde protein covalent bonds in vitro (45). Methylene blue may inhibit the formation of aldehyde adducts at various levels: (*a*) aldehyde formation, (*b*) adduct formation, and (*c*) adduct stability. This decrease in aldehyde adducts formation may be important in the decreased expression of the collagen gene.

MDA- and 4-hydroxynonenal (4-HNE) protein adducts have been demonstrated in ascorbic acid–treated cultured fibroblasts, and their formation is inhibited with *d*-α-tocopherol (11). Presumably, by inhibiting lipid peroxidation and thus the formation of reactive aldehydes, such as MDA and 4-HNE, *d*-α-tocopherol inhibits adduct formation. Presently, no other biologically important function has been ascribed to *d*-α-tocopherol distinct from its role as an antioxidant (46). *d*-α-Tocopherol may alter the expression or function of putative *trans*-acting factors or modify the transport of these DNA binding proteins into the nucleus. This latter effect could result from altered membrane fluidity induced by an increased *d*-α-tocopherol content in the lipid bilayer (47). It is also important to note that aldehydes may form adducts with virtually all cellular elements, including DNA (44, 45). Thus, it is conceivable that aldehydes may stimulate collagen gene expression, by a direct interaction with DNA *cis*-acting regulatory elements. Preliminary results of transfection experiments suggest that the "lipid peroxidation–responsive element" is not present within the 2,500 bp upstream from the initiation site of transcription or in the first intron of the collagen α_1 (I) gene (Houglum, K., unpublished observations). Additional studies are required to determine if the "lipid peroxidation–responsive element" is present as an enhancer located elsewhere in the collagen α_1 (I) gene.

There are only a few examples of regulation of gene expression by oxidative stress including *oxy*R and heme oxygenase genes (48–50). The *oxy*R gene is required for the stimulation of a group of genes by hydrogen peroxide in *Salmonella typhymurium* and *Escherichia coli* (48). The oxidized, but not the reduced, *oxy*R protein activates transcription of these oxidative stress–inducible genes in vitro (49). Likewise, oxidative stress, but not heat shock, induced heme oxygenase in cultured human fibroblasts, suggesting that heme oxygenase may constitute a cellular defense mechanism against oxidative damage (50). Whether mechanisms similar to those of *oxy*R and heme oxygenase induction are responsible for the modulation of constitutive collagen expression by lipid peroxidation remains to be elucidated.

It is not known whether the mechanisms that modulate constitutive collagen gene expression in cultured cells also occur in vivo. Cells are usually exposed to a higher oxygen tension in vitro (51) than in vivo (52) and this could induce high levels of basal lipid peroxidation, which in turn may lead to a high constitutive collagen gene expression. When myocardial cells are exposed to higher oxygen tensions they have a parallel increase in lipofuscin (53) which is thought to be a product of lipid peroxidation (54). However, under the conventional incubation conditions (95% air/5% CO_2) used in our experiments, and in the absence ascorbic acid, the level of lipid peroxidation found in our cultured human fibroblasts is comparable with that in cultured myocardial cells (280±30 pmol/mg protein) (55) and in normal skin (21, 22) and other tissues in vivo (Houglum, K., unpublished observations; 19, 20, 29). These findings suggest that the degree of lipid peroxidation is comparable in normal cultured cells and normal tissues in vivo.

Another important issue is whether the constitutive collagen gene expression is spuriously high in cultured fibroblasts. Several studies seem to indicate that collagen gene expression is quantitatively and qualitatively similar in vitro and in vivo. For example, the rates of collagen production relative to total protein in cultured fibroblasts (11, 13, 15, 27, 56) are in the same range as in normal skin in vivo (57). Although absolute rates of collagen production are difficult to measure the data available suggest that the values are equivalent (1–3 μmol proline/mg protein per h) in normal cultured fibroblasts including the cells utilized in this study (56, and calculated from reference 27) and in normal skin in vivo (58, and estimated from reference 59). Also, the steady-state level of collagen α_1 (I) mRNA by solution hybridization is in the same range in cultured fibroblasts and in normal rodent skin in vivo (Buck, M., unpublished observations). Furthermore, the level of expression of the collagen α_1 (I) gene is high in mouse tendon fibroblasts in vivo (9) and in mouse fibroblasts in vitro (60). Lastly, the rates of collagen degradation are apparently equivalent in cultured fibroblasts (27, 61) and in normal skin in vivo (58, 59). It should be emphasized that whether or not the modulation of constitutive collagen gene expression by lipid peroxidation is physiologically important, it could be a potentially complicating variable in studies of collagen gene transcription in cultured fibroblasts, and possibly in other cultured cells.

Whether lipid peroxidation plays a role in the modulation of constitutive collagen gene expression in vivo is unknown and will require further study. Due to the low toxicity of *d*-α-tocopherol and methylene blue, the modulation of constitutive collagen gene expression by lipid peroxidation could now be assessed in normal animals.

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