Regulation of Type I Collagen mRNA by Amino Acid Deprivation in Human Lung Fibroblasts

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The steady state levels of α1(I) collagen mRNA are decreased by retinoic acid and prostaglandin E₂. These effector substances decrease the uptake of A system amino acids. We examined the effect of amino acid deprivation on the steady state levels of α1(I) collagen in human lung fibroblasts. Maintenance of fibroblasts in amino acid-free medium decreased α1(I) collagen mRNA levels by 29% at 24 h and 78% at 72 h. Frequent refeeding of cultures with amino acid-free medium resulted in more rapid decreases in intracellular amino acids and in α1(I) collagen mRNA levels. The decrease in α1(I) collagen mRNA levels was mediated by decreases in mRNA stability as assessed by a half-life determination using actinomycin D and by decreases in the rate of transcription as assessed by nuclear run-on assay. Treatment of fibroblasts with medium containing amino acids resulted in rapid restoration of α1(I) collagen mRNA levels. This increase in α1(I) collagen mRNA expression required protein synthesis as determined by cycloheximide sensitivity and was inhibited by prostaglandin E₂. These data indicate that α1(I) collagen mRNA levels are sensitive to alterations in the amount of intracellular amino acids and suggest a potential mechanism whereby α1(I) collagen accumulation may be regulated independent of inflammatory mediators following lung injury.

The biosynthesis of type I collagen is complex and involves both intracellular and extracellular sites of regulation. The accumulation of type I collagen by lung fibroblasts is increased by transforming growth factor-β and insulin and is decreased by prostaglandin E₂ (PGE₂), retinoic acid, and interferon-γ (1–7). We and others previously reported that both PGE₂ and retinoic acid induce large decreases in the expression of α1(I) collagen mRNA by these cells (2–4). These effects require protein synthesis and are mediated by decreases in the rate of transcription of the α1(I) collagen gene as well as by decreases in the stability of the α1(I) collagen mRNA.

PGE₂ and retinoic acid decrease the uptake of neutral amino acids transported by A system amino acids in lung fibroblasts (7, 8). The activity of the A system is Na⁺-dependent and is responsive to hormones, extracellular amino acids, and other effector substances (9, 10). The decreases in amino acid transport were rapid and occurred within 1 h of exposure to the effector substance. In contrast, the decreases in the steady state levels of α1(I) collagen mRNA occurred between 8 and 12 h following exposure. This kinetic relation whereby decreases in amino acid uptake preceded decreases in collagen mRNA levels suggests that depletion of amino acid levels are associated with decreases in α1(I) mRNA levels. Certain other mRNAs are regulated by amino acid availability. For example, asparagine synthetase mRNA is up-regulated by amino acid starvation, whereas β-actin and glyceraldehyde 3-phosphate are decreased in abundance (11–13).

In these studies, we examined the relation between amino acid depletion and α1(I) collagen mRNA levels in human lung fibroblasts. We find that amino acid depletion caused marked decreases in α1(I) collagen mRNA levels that rapidly reaccumulate following the addition of amino acids. This decrease in α1(I) collagen mRNA was mediated by decreasing the rate of transcription of the α1(I) collagen gene and by decreasing the stability of the mRNA.

MATERIALS AND METHODS

Cells and Tissue Cultures—Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in Dulbecco’s modified Eagle’s medium with 0.37 g of sodium bicarbonate/100 ml, 10% fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, and 0.1 mM nonessential amino acids. After confluence was reached, the serum content of the medium was reduced to 0.4%. The numbers of cells were determined by triplicate cell counts with an electronic particle counter (Coulter Counter ZM).

RNA Isolation and Northern Analysis—Total cellular RNA was isolated by the single-step method employing guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi (14). RNA was quantitated by absorbance at 260 nm. Purity was determined by absorbance at 280 and 310 nm. RNA (10 μg) was electrophoresed on a 1% agarose, 6% formaldehyde gel and transferred to a nitrocellulose filter. RNA loading was assessed by ethidium bromide staining of ribosomal bands fractionated on agarose-formaldehyde gels and by co-hybridization with Gs, which is a constitutively expressed mRNA that codes for a GTP-binding protein (15). Hybridization was performed using 0.5–1.0 × 10⁶ cpm/lane labeled probe (specific activity, 4–10 × 10⁶ cpm/μg), and the filter was washed according to methods described by Thomas (16). The filter was exposed to X-ray film for autoradiography at several different times to ensure that the bands could be quantified by densitometry within the linear range. The loading was verified by probing with an oligonucleotide that specifically identifies the 18S ribosomal subunit (17). The probes utilized in these experiments were a rat cDNA α1(I) collagen clone (18) that specifically identifies corresponding human α1(I) mRNA, G₁β, a GTP-binding protein (kindly provided by Dr. R. Reed, John Hopkins University School of Medicine) (15), and cyclooxygenase-1 (19).

Assessment of Free Amino Acid Pools—Quiescent fibroblast cultures were incubated with fresh medium containing no amino acids at 37 °C for the indicated time periods. Following the incubation, the medium or the cellular material was extracted with 1 ml of 10% trichloroacetic acid. The amount of free amino acids in the supernatant was determined by using actinomycin D and by decreases in the rate of transcription of the α1(I) collagen gene as well as decreases in the stability of the mRNA.

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Quiescent fibroblast cultures were untreated or cultured in amino acid-deficient medium for 48 h. The medium was removed and replaced with medium without amino acids (AAF) or medium containing the full complement of 15 amino acids (AAR). After 24 h, the RNA was extracted, electrophoresed, and transferred to nitrocellulose. The filter was probed with cDNA for α1(I) collagen and an oligonucleotide that identifies the 18 S ribosomal subunit. After probing, an autoradiogram was obtained, and densitometry was performed.

FIG. 1. The effects of amino acid deficiency on α1(I) collagen mRNA levels. Quiescent fibroblast cultures were maintained in amino acid-deficient medium without serum for 48 h. The medium was replaced with amino acid-free medium (AAF) or medium containing the full complement of 15 amino acids (AAR). After 24 h, the RNA was extracted. A, the filter was stripped and reprobed for Gs. The filter was stripped and reprobed for α1(I) collagen mRNA and finally reprobed with an oligonucleotide that identifies the 18 S ribosomal subunit. B, the filter was stripped and reprobed with cDNA for α1(I) collagen. The filter was stripped and reprobed for cyclooxygenase-1 (Cox 1) mRNA and finally reprobed with an oligonucleotide that identifies the 18 S ribosomal subunit.

Nuclear Run-on Assay—Medium was removed from 150-mm dishes, and the cells were washed twice with Puck’s saline and scraped into a Nonidet P-40 lysis buffer. Following two low speed spins, the pellet was reconstituted in a glycerol buffer. In vitro labeling of nascent RNA and hybridization with cDNAs immobilized on nitrocellulose filters were performed according to the methods outlined by Greenberg and Ziff (20) and Groudine et al. (21). No hybridization occurred to filters containing plasmids without inserts.

Statistics—A Student’s t test was used for means of unequal size (22). Probability values <0.05 were considered significant.

RESULTS

Quiescent confluent lung fibroblasts were maintained in medium without amino acids to deplete intracellular amino acids. After varying periods of time, we determined the steady state levels of α1(I) collagen mRNA. Gel loading was assessed by ethidium bromide staining and by probing the filter with an oligonucleotide that recognizes the 18 S ribosomal subunit. The levels of α1(I) collagen mRNA were slightly decreased (29 ± 7%, mean ± S.E., n = 3) in fibroblasts maintained in amino acid-deficient medium for 24 h. Maintenance in amino acid-free medium for 72 h resulted in a large decrease in α1(I) collagen mRNA levels (Fig. 1). The results of five such experiments revealed that amino acid deprivation for 72 h decreased α1(I) collagen mRNA levels by 78% ± 5 (mean ± S.E.).

The addition of the usual complement of 15 amino acids present in complete medium restored α1(I) collagen mRNA levels, indicating that the absence of added amino acids was not toxic to the cells (Fig. 1). The availability of amino acids markedly affected α1(I) collagen mRNA levels, but only minimally affected the steady state levels of Gs mRNA (which encodes a GTP-binding protein) and cyclooxygenase-1 mRNA (Fig. 2). The amino acid-mediated induction of α1(I) collagen mRNA levels required protein synthesis. Cycloheximide alone at 5 μm inhibits protein synthesis by greater than 90% (23) and does not affect α1(I) collagen mRNA levels in these cells (24) and data not shown). However, the presence of cycloheximide blocked the amino acid-induced increase in α1(I) collagen mRNA levels (Fig. 3).

We examined the kinetic relation between the addition of amino acids and the reexpression of α1(I) mRNA. Quiescent fibroblasts were maintained in medium with or without amino acids for 48 h. Northern analysis was performed on amino acid-deficient cells that were reinfused with serum-free medium containing amino acids. The steady state level of α1(I) collagen mRNA was increased at 8 h (approximately 4-fold as assessed by densitometry of the two major α1(I) collagen mRNA signals) and further increased at 24 h following refeeding with medium containing amino acids (Fig. 4).
To determine whether frequent refeeding with amino acid-free medium increased the depletion of intracellular amino acids by increasing efflux and preventing reuptake, we determined the amount of free amino acids in the medium and in the intracellular compartment. The fibroblasts were placed in amino acid-deficient medium, and the amount of A system amino acids in the medium was determined after 6 h. We found that glycine, alanine, and lesser amounts of proline accumulated in the medium by efflux (Fig. 5). Small amounts of leucine transported by the L system were also detected in the medium.

The intracellular concentration of the A system amino acids glycine, proline, and alanine and the L system amino acid leucine was determined in cells incubated in medium with or without amino acids. The intracellular levels of the amino acids were variably decreased. Glycine decreased by 17%, proline by 64%, alanine by 71%, and leucine by 88% after 12 h (Fig. 6). When the medium was changed at 4 and 8 h to remove amino acids accumulating in the extracellular space from efflux (and therefore potentially available for reuptake), the intracellular levels of glycine, proline, and alanine decreased further, whereas leucine levels were unchanged.

We examined the effect of rapidly altering the levels of amino acids by frequent refeeding with amino acid-deficient medium on \( \alpha_1(1) \) collagen mRNA levels. Rapid depletion of intracellular amino acids was accomplished by refeeding the cells every 5 h (four changes) during the 24-h period with amino acid-deficient medium. We assessed the steady state levels for \( \alpha_1(1) \) collagen and \( \alpha_2(1) \). After 24 h, we found that \( \alpha_1(1) \) mRNA levels fell by 71% in cells frequently refed with amino acid-free medium (Fig. 7). In contrast, the level of \( \alpha_1(1) \) mRNA decreased only 39% in cells cultured in amino acid-deficient medium but not frequently refed.

To examine the mechanism whereby amino acid depletion decreased collagen mRNA levels, we determined the half-life for \( \alpha_1(1) \) collagen mRNA and the rate of transcription of the \( \alpha_1(1) \) gene. We found that amino acid depletion decreased the stability of the \( \alpha_1(1) \) mRNA (Fig. 8). The half-life of the mRNA was assessed by measuring the decay of the mRNA after the addition of actinomycin D. Linear regression analysis using the results of three such experiments employing a variety of time points (2.5, 5, 7, and 9 h) revealed that amino acid depletion decreased the half-life of the \( \alpha_1(1) \) mRNA from 9.7 to 4.3 h.

**Fig. 5.** The accumulation of amino acids in the medium after addition of amino acid-free medium. The medium was removed, cells were rinsed three times with Puck's saline, and medium without amino acids was added. After 6 h, the medium was removed, and the levels of glycine, alanine, proline, and leucine in the medium were determined by amino acid analysis.

**Fig. 6.** The intracellular levels of amino acids after addition of amino acid-free medium. The medium was removed, and cells were rinsed three times with Puck's saline and replaced with complete medium (CM) or medium without amino acids (AAF). In additional dishes, the medium without amino acids was replaced after 4 and 8 h (AAF R). After 12 h, the medium was removed, and the intracellular levels of glycine, alanine, proline, and leucine were determined by amino acid analysis.


Because this decrease in half-life cannot account entirely for the decreases in α1(I) mRNA following amino acid deprivation, we assessed the rate of transcription by a nuclear run-on assay. Nuclei were isolated from fibroblasts maintained in medium with or without amino acids for 48 h. The rate of transcription of the α1(I) gene was reduced by 73% (mean of two experiments) in nuclei obtained from fibroblasts maintained without amino acids. In contrast, the rate of transcription for Gs was unchanged. The addition of amino acids restored the transcription rate for α1(I) collagen (Fig. 9).

We examined the effect of PGE₂ on levels of intracellular amino acids and on α1(I) collagen levels in cells deprived of amino acids. The addition of PGE₂ at 10⁻⁷ M to cultures maintained in amino acid-free medium for 8 h further decreased the intracellular levels of glycine by 35% to 75.4 ± 3.5 mmol/10⁶ cells and proline by 20% to 29.5 ± 1.1 mmol/10⁶ cells as compared with cells maintained in amino acid-free medium. PGE₂ at 10⁻² M also induced a small additional decrease in α1(I) mRNA levels in cells maintained in amino acid-deficient medium (Fig. 10). When fibroblasts were reexposed to amino acids, PGE₂ inhibited the restoration of α1(I) mRNA levels by 76 ± 7% (mean ± S.E., n = 3).

**DISCUSSION**

We found that the steady state level of collagen α1(I) mRNA was decreased by treatment of fibroblasts with amino acid-deficient medium. In contrast, changes in amino acid availability minimally affected the levels of the constitutively expressed cyclooxygenase-1 and Gs mRNAs. The decrease in α1(I) mRNA levels was dependent on the time of exposure to amino acid-deficient medium and the rate of decrease in intracellular amino acids. Short exposures to amino acid-deficient medium resulted in a 29% decrease in mRNA levels, and prolonged exposure resulted in a 78% decrease. When the intracellular levels of amino acids were rapidly lowered by frequent refeeding with amino acid-deficient medium, the α1(I) mRNA levels decreased more markedly. Frequent refeeding decreased intracellular amino acids by removing amino acids appearing in the medium by efflux and preventing reuptake. The decrease in α1(I) collagen mRNA levels was not the result of toxicity because restoring amino acids to the medium resulted in a rapid restoration of mRNA levels.

Alteration of intracellular amino acid levels affects the steady state levels of several other mRNAs (9-11, 25). The steady state mRNA level for asparagine synthetase is up-regulated by amino acid derivation in baby hamster kidney cells (11, 12). A novel cis-acting element (5'-CATGATG-3') located in the proximal asparagine promoter mediates this effect and binds nuclear proteins (26). However, analysis of the promoter region of the α1(I) collagen gene did not reveal a similar binding site. Other examples of amino acid-regulated mRNAs include Cu-Zn superoxide dismutase, glyceraldehyde 3-phosphate, and histone H2. These mRNAs are decreased by amino acid starvation in rat Fao hepatoma cells (13). In addition, insulin-like growth factor I mRNA levels in hepatocytes are decreased by amino acid deprivation and increased by excess (27).
Certain effector substances regulate gene expression by altering intracellular amino acid levels. Interferon-γ decreases expression of collagenase and stromelysin by decreasing the intracellular concentration of tryptophan (25, 28). Interferon-γ also decreases production of α(I) collagen mRNA (29). However, we found that the addition of tryptophan to amino acid-deficient medium did not restore α(I) mRNA levels (unpublished results) suggesting that one or more other amino acids are necessary to induce this affect.

Depletion of amino acids decreased the rate of transcription of the α(I) collagen gene and the stability of the mRNA. This process was relatively selective because the rate of transcription of Gc was unchanged by decreased amino acid availability. The molecular mechanism whereby amino acid depletion causes alterations in gene expression and mRNA stability is unknown. The sensing mechanism may involve changes in the level of aminoacylation of tRNA. For example, the rate of protein breakdown in histidine starvation of the Chinese hamster ovary cell is regulated by the levels of aminoacylation of tRNA (30). Studies employing temperature-dependent mutant cell lines suggest that regulation of asparagine synthetase activity may involve tRNA acylation (31, 32). This regulatory mechanism may also affect mRNA levels of selected genes, possibly by altering the production of a transcriptional activator under circumstances of amino acid deprivation. Our results employing cycloheximide also suggest that synthesis of one or more specific proteins (perhaps a transcription factor) is initially required for activation of α(I) collagen mRNA levels by amino acid exposure. We find that cycloheximide inhibited the induction of α(I) collagen mRNA following the addition of amino acids to amino acid-deficient cells.

Our previous results showed that PGE₂ and retinoic acid decreased the transport of A system amino acids by increasing the Km of the A system transporter (7, 8). Both of these effectors decrease the rate of transcription of the α(I) collagen gene. Our results employing amino acid-deficient medium suggest that the PGE₂-induced decreases in α(I) collagen mRNA levels may be mediated through decreases in intracellular amino acids. PGE₂ further decreased α(I) collagen levels when fibroblasts were cultured in amino acid-deficient medium, perhaps by inhibited reuptake of amino acids accumulating in the medium by efflux. In addition, PGE₂ blocked the restoration of α(I) mRNA levels following reexposure to amino acids. We previously found that PGE₂ was more effective in inhibiting α(I) collagen accumulation induced by transforming growth factor-β than that induced by insulin (33). Because insulin is a strong inducer of amino acid transport, it may inhibit the effect of PGE₂ by directly stimulating uptake.

Alterations in amino acid availability may affect α(I) collagen mRNA expression by fibroblasts residing within a bioma-trix and in situ. The steady state levels of α(I) collagen mRNA decreases in fibroblasts that are placed within collagen gels, perhaps as a result of matrix-fibroblast interactions that decrease the availability of nutrients by alternations in transporter function (34–36). It is noteworthy in this regard that suspension of 3T3 fibroblasts in methylenecollodene decreases the rate of transcription of the α(I) collagen gene and decreases the stability of the α(I) collagen mRNA (37). In the lung, fibroblasts that reside in the pulmonary interstitium do not express detectable levels of α(I) mRNA as assessed by in situ hybridization (38). Following lung injury, α(I) collagen levels rapidly increase, presumably from exposure to fibrogenic cytokines such as transforming growth factor-β. However, alteration in vascular permeability and proteolytic disruption of the extracellular matrix may provide greater access for amino acids into the interstitium, resulting in fibroblast activation.

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