The Effect of Retinoic Acid on Amino Acid Uptake and Protein Synthesis by Lung Fibroblasts*

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The effect of retinoic acid (RA) on the uptake and utilization of extracellular amino acids by fetal lung fibroblasts was examined. RA decreased the incorporation of [14C]proline into collagen and other proteins. The effect was maximal at a RA concentration of 10^{-5} M; smaller decreases were observed at a RA concentration of 10^{-4} M. This decrease in collagen formation was associated with a large decrease in intracellular [3H]proline. The decrease in intracellular [3H]proline was first observed at 2 h following the addition of RA to cell cultures. Transport studies employing radiolabeled amino acids revealed that RA decreased the uptake of proline, 2-aminoisobutyric acid, and 2-(methylamino)isobutyric acid but not leucine or methionine. Kinetic analysis of 2-aminoisobutyric acid uptake indicated that this effect was mediated primarily by an increase in apparent K_m, with a lesser decrease in V_{max}. RA-induced inhibition of proline uptake was not abolished by the presence of cycloheximide nor by pretreatment with indomethacin. Naa,Ka-ATPase activity was not affected by RA treatment. These results suggest that RA modulates protein production in fibroblasts by altering the function of the Na^+-dependent A transport system for amino acid uptake.

Retinoic acid (RA) exerts profound effects on the development and homeostatic properties of many endodermal, mesodermal, and ectodermal-derived tissues (1). Applied to the human skin, it increases fragility and decreases the size of keloids (2, 3). Retinoids also have been used to protect skin and other tissues from malignant transformation (4, 5). In cultured cells, RA regulates the production of several extracellular matrix components including decorin, biglycan, and collagen types I and IV (6–8). In fibroblast cultures, RA selectively decreases collagen production and the steady state level of a2(I) collagen mRNA (7). This effect could be mediated by nuclear retinoic acid receptors or via alterations in the lipid bilayer of membranes (9, 10).

RA can induce both direct and indirect alterations in membrane function. Several genes encoding cell-surface receptors are regulated by RA (11). For example, RA at micromolar concentrations up-regulates the number of sites but not the affinity of the melanocyte-stimulating hormone receptor (12). RA also down-regulates expression of the epidermal growth factor receptor by inhibiting promotor activity in the 5'-flanking region of the receptor gene (13). As lipophilic agents, retinoids localize to phospholipid bilayers and induce structural changes in biomembranes (10, 14, 15), such as restriction of acyl chain motion (16). These alterations may affect lipid-protein and lipid-lipid interaction and the fluid state of the membrane (10, 17, 18). Retinoids are reported to increase the surface area of membranes (19, 20) as well as the cell-to-substrate adhesiveness (21). Such alterations in membrane structure could affect the transport of amino acids into the cell.

The uptake of neutral amino acids into fibroblasts is mediated by several partially overlapping transport systems. The A system is Na^+-dependent and subject to regulation by hormones and other effector substances (22–24). For example, prostaglandin E_2 (PG_E_2) decreases A system activity in lung fibroblasts in culture (25). Other transport agencies including the L and ASC systems are less affected by exogenous factors. In order to further examine the effect of RA on protein accumulation, we assessed the effect of retinoic acid on collagen production and the transport of amino acids in fibroblast cultures. We found that RA-induced decreases in collagen formation were associated with decreased intracellular levels of free proline and large decreases in the activity of the Na^+-dependent A transport system for amino acids.

MATERIALS AND METHODS

Cells and Cell Culture—Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in Dulbecco’s modified Eagle’s medium with 0.57 g of sodium bicarbonate/100 ml, 10% fetal bovine serum, 100 units of penicillin/ml, 10 μg of streptomycin/ml, and 0.1 mM nonessential amino acids. The cells were maintained in a humidified 5% CO_2 in 35-mm dishes (Falcon, Oxford, CA). After confluence was reached, the cells were made quiescent by reducing the serum content to 0.4%. All experiments were performed under serum-free conditions. Cell number was determined by duplicate cell counts with an electronic particle counter (Coulter Counter ZM).

Reagents—All-trans-RA (Sigma) was reconstituted in 100% ethanol. All procedures involving the use of RA were performed in subdued light. Control cultures were treated with equivalent concentrations of ethanol, which never exceeded 0.1% (w/v). Indomethacin (Sigma) was dissolved in ethanol, evaporated to dryness with nitrogen, and resuspended in culture medium. The medium was filtered through a Millipore filter and added to the appropriate culture plates. 4-[14C]Proline (102 Ci/mmol), 4-[3H]Leucine (46 Ci/mmol), and 2-amino[1-14C]isobutyric acid (58 mCi/mmol) were purchased from Amersham Corp. and 2-(methylamino)[1-14C]isobutyric acid (50 mCi/mmol) was purchased from DuPont-New England Nuclear.

Polyacrylamide Gel Electrophoresis (PAGE)—Confluent quiescent fibroblast cultures were labeled in serum-free medium containing

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solution of protease inhibitors yielding a final concentration of 10⁻⁴ M phenylmethylsulfonyl fluoride, 10⁻³ M hydroxymercuribenzoate, and 2 × 10⁻³ M EDTA. The samples were dialyzed against H₂O at 4°C, lyophilized, and digested with pepsin at 4°C overnight. PAGE was performed on a 7.5% gel (26). Autoradiography was performed according to the method of Bonner and Laskey (27). The identity of the collagen bands was confirmed by co-migration with pepsin-treated radiolabeled type I collagen and collagenase sensitivity. For protein synthesis experiments, the cell cultures were maintained under identical conditions with the exception of using [³⁵S]methionine rather than [³H]proline.

Protein Determination—The cell monolayer was extracted with 10% trichloroacetic acid, and the trichloroacetic acid-insoluble material was solubilized by digesting the material in 10% trichloroacetic acid at 60°C for 1 h. An aliquot of the digested material was assessed for radioactivity by scintillation counting. The trichloroacetic acid-soluble and trichloroacetic acid-insoluble fractions were analyzed for radiolabeled proline and tricholine. Following hydrolysis in 6 N HCl at 110°C for 20 h, the amount of nondialyzable [³H]proline and the amount of total proline (radiolabeled and nondialyzed) was determined on an automatic amino acid analyzer fitted with a stream-splitting device and a fraction collector. To determine the amount of nondialyzable [³H]proline, the appropriate fractions were placed in scintillation fluid, and radioactivity was assessed. To determine the nanomoles of proline in the hydrolysate, the analyzer added ninhydrin to the sample and assayed for amino acids colorimetrically.

Transport of Amino Acids—Quiescent fibroblast cultures were incubated with fresh medium containing labeled amino acids and were incubated at 37°C for the indicated time periods with fresh medium (25). The incubation was terminated by rinsing the cells three times with cold Puck’s saline. Nonspecific binding was determined by immediately removing the medium containing the labeled amino acid. Following the incubation, the cellular material was extracted with 1 ml of 10% trichloroacetic acid. The amount of trichloroacetic acid-soluble labeled amino acids were determined by scintillation counting. The trichloroacetic acid-insoluble fraction was used for protein determinations as described above. The initial rate of influx was estimated by 30- to 3-min incubations in medium containing the labeled amino acid. Efflux was estimated by loading the monolayers with [³⁵S]AIB for 1 h and then measuring by scintillation counting the initial rate of solute escape into fresh serum-free medium containing no AIB or with RA (28). The incubation was terminated by removing the medium and washing the cells with ice-cold phosphate-buffered saline, pH 7.4.

Isolation of Plasma Membranes—RA treated and nontreated fibroblast cell plasma membranes were isolated by modification of previously described methods (29). Plasma membranes were obtained by rinsing fibroblast cell monolayers with 1-2 ml of 10 mM Tris·HCl, pH 7.0, and harvesting the cells with a rubber policeman. The cells were then suspended in 10 mM Tris·HCl buffer, pH 7.0, allowed to swell for 15 min, and gently homogenized in a tight-fitting Dounce homogenizer about 20 strokes. This homogenate was centrifuged at 680 × g for 1 min at room temperature. The supernatant was centrifuged at 8000 × g for 20 min at 2°C to sediment the whole plasma membranes. The pellet constituting a crude plasma membrane fraction was resuspended in medium at 4°C, at a final protein concentration of 4.8-5.6 mg/ml and stored at -70°C.

Na⁺,K⁺-ATPase Assay—To determine ATPase activity, the membranes were preincubated at 37°C for 10 min in 0.475 ml of a medium containing 100 mM NaCl, 20 mM KCl, 50 mM Tris·HCl, pH 7.4, at 37°C, 5 mM NaN₃, 3 mM MgCl₂, and 0.5 mM EDTA. The reaction was started by addition of 0.025 ml of ATP to a final concentration of 2 mM, and was continued for 60 min at 37°C, and then terminated with 0.2 ml of ice-cold 3% sodium acetate, pH 4.3. Basal Mg²⁺-ATPase activity was determined in the presence of 0.2 mM ouabain. This concentration of ouabain inhibited Na⁺,K⁺-ATPase activity by greater than 90%, as reported by others (30, 31). Na⁺,K⁺-ATPase activity was calculated as the difference between the total ATPase and Mg²⁺-ATPase activities. Mg²⁺-ATPase activity in the plasma membranes preparations was -10% of corresponding Na⁺,K⁺-ATPase activity.

Statistics—A Student’s t test was used for means of unequal size (32). Probability values <0.05 were considered significant.

**RESULTS**

Fibroblasts were treated with all-trans-RA for 24 h in the presence or absence of labeled amino acid. Retinoic acid treatment caused a concentration-dependent decrease in the amount of type I collagen production by quiescent human lung fibroblasts in culture as determined by PAGE analysis of pepsin-digested medium proteins (Fig. 1A). Densitometric analysis demonstrated that type I collagen levels were reduced 90% by RA at 10⁻⁵ M, by 72% at 10⁻⁶ M, and by 22% at 10⁻⁷ M. The dose-response relationship established by several similar experiments is shown in Fig. 1B. The cell morphology of cultured cells was unaffected by exposure to all-trans-RA (10⁻⁵ M) or 0.1% ethanol as determined by phase-contrast microscopy. In addition, RA treatment for ≤24 h did not alter cell number (data not shown).

The decreases in [³H]proline labeling of collagen and other proteins suggested that all-trans-RA treatment (10⁻⁵ M) affected the intracellular [³H]proline pool. After various time periods following the addition of retinoic acid and [³H]proline, the cells were extracted with 10% trichloroacetic acid, and the amount of radiolabel in the trichloroacetic acid-soluble and trichloroacetic acid-insoluble fractions were determined.
(Table I). The amount of [3H]proline in the trichloroacetic acid-soluble cellular fraction was slightly decreased at 2 h (16%), moderately decreased at 6 h (45%), and maximally decreased at 24 h (64%) as compared with untreated controls. The amounts of [3H]proline radiolabeled proteins were decreased at the various time periods (Table I).

The production of proteins was assessed by amino acid analysis after 24 h of RA treatment. The total proline content of both extracellular and intracellular proteins was reduced by approximately 50% in RA-treated fibroblasts. The specific activity of [3H]proline incorporated into intracellular proteins was decreased by treatment with RA (238.7 ± 10.7 cpm/nM, mean ± 1 S.E., in untreated and 36.7 ± 2.3 in RA-treated cultures).

To determine whether RA-induced decreases in [3H]proline labeling of proteins was specific for this amino acid, the cultures were radiolabeled with [35S]methionine for 8 h following RA treatment. The incorporation of radiolabeled methionine into proteins was not decreased by treatment with RA and the relative proportions of proteins synthesized was not altered (Fig. 2), indicating that RA did not initially affect overall protein synthesis.

The decrease in proline pool size suggested that retinoic acid was affecting the transport of specific amino acids. Proline uptake was assessed after treatment of fibroblasts with RA for 2 h. The initial rate of [3H]proline uptake was reduced by 50% in RA-treated cultures (Fig. 3A). The incorporation of [3H]proline into proteins during the 20-min labeling period was reduced by 78% (Fig. 3B). This effect of RA on amino acid uptake was reversible (Fig. 4). Removing the RA-treated medium after various treatment periods and refeeding with fresh control medium reversed the effect of RA on amino acid uptake. The uptake in RA-treated cultures returned to within 10% of untreated values after 24 h in control medium.

The transport of proline is mediated by the Na+-dependent A transport system (22). The effect of RA on the Na+-dependent A transport system was evaluated by using the amino acid analogue, AIB. This amino acid is not metabolized (33). The pretreatment of quiescent fibroblasts with RA for 8 h resulted in a decrease in [14C]AIB uptake. The initial rate of uptake of [14C]AIB was decreased by approximately 52% following addition of the amino acid (Fig. 5A). RA did not affect the function of the Na+-independent L transport system as assessed by the uptake of [3H]leucine. RA also did not affect the uptake of methionine, an amino acid that can be transported by either the A or the L system (data not shown).

To verify that AIB was transported by the A and not the ASC or L transport systems, we employed 2-(methylamino)isobutyric acid (MeAIB). This N-methylated derivative of AIB is selectively transported by the A system (22). RA inhibited the initial rate of entry for MeAIB by 55%, confirming that the decreases in both MeAIB and AIB were mediated by the A system (Fig. 5B). Treatment with RA also affected the efflux of [14C]AIB. RA treatment decreased the rate of efflux by 26% (Fig. 6).

We determined whether the effect of RA on amino acid transport was dependent on protein synthesis or prostaglandin release. Treatment of the cultures with cycloheximide at 5 μM (to inhibit protein synthesis) resulted in no change in the uptake of [3H]proline (Fig. 7). The effect of the combination of RA and cycloheximide on amino acid uptake was similar to that induced by RA alone. Pretreatment with indomethacin (to inhibit prostaglandin release) did not affect the inhibition of [3H]proline uptake by RA.

In order to further investigate the mechanism of retinoic acid action, kinetic studies were performed to evaluate the effect of RA on the uptake of [14C]AIB. Kinetic analysis of AIB uptake in untreated and RA-treated cultures showed that RA treatment resulted in a 3-fold increase in the apparent Vmax and a small decrease in Km as illustrated in Fig. 8. The combination of this increase in apparent Km and a decrease in Vmax accounted for the large decrease in amino acid uptake.

Na+-dependent amino acid uptake is affected by the Na+ gradient established by the Na+,K+-ATPase (34–37). We examined the Na+,K+-ATPase activity in membranes isolated from untreated and RA-treated cells to determine whether RA directly affected the Na+,K+-ATPase. We found that Na+,K+-ATPase activity was similar in untreated and RA-treated membranes. Enzymatic activity was 581 nmol of P/
the transport was mediated primarily by increases in the apparent $\text{Pi/h/mg}$ of protein for the RA-treated preparation. In separate cultures, the medium was removed and the cultures were refed with media containing 0.4% serum without RA. After 24 h, the uptake of [3H]proline was determined. The data are expressed as mean ± 1 S.E.; $n = 3$. The velocity of proline uptake in untreated culture at 24 h representing 100% was 68 pmol/min/mg of protein.

$K_m$ for the transporter. PGE$_2$ actually increased $V_{max}$ slightly. The decrease in transport induced by RA was also mediated primarily by increases in the apparent $K_m$. In contrast to PGE$_2$, the RA induced both an increase in $K_m$ and a decrease in $V_{max}$. These alterations in transporter function resulted in larger decreases in the rate of transport than that induced by PGE$_2$. The efflux studies indicate that this effect appears to be bidirectional (28). The decreases in the rate of transport was not the result of synthesis of a protein which either modifies or degrades the transporter because cycloheximide did not inhibit RA actions on amino acid uptake. In addition, RA did not function through PG release because the addition of indomethacin did not affect RA induced inhibition of proline transport.

The decreases in [3H]proline transport resulted in minimal labeling of proline-rich collagen. In contrast, overall protein synthesis was not affected for at least 8 h as determined by [35S]methionine labeling. The intracellular proline pool is derived primarily from exogenous sources but the degradation of proteins can provide an alternate source for this amino acid, particularly during conditions of nutritional deprivation (41). When exogenous proline is removed from the media, the intracellular concentration of proline rapidly decreases (28). The primary source of amino acids for charging tRNAs is not the result of synthesis of a protein which either modifies or degrades the transporter because cycloheximide did not inhibit RA actions on amino acid uptake. In addition, RA did not function through PG release because the addition of indomethacin did not affect RA induced inhibition of proline transport.

DISCUSSION

The addition of retinoic acid to human embryonic lung fibroblast cultures inhibited the uptake of proline, AIB, and MeAIB by the A system for amino acid transport. This effect required only short term exposure to RA (2 h or less) and did not require protein synthesis. The A transport system is Na$^+$-dependent, accepts N-methylation of amino acid substrates, and mediates the transport of linear aliphatic amino acids and the imino acid proline. Its activity is modified by hormones (38), growth factors (39), nutritional conditions (40), and the electrochemical potential across the cell membrane (36, 37).

We reported previously that PGE$_2$ inhibits the activity of the A system for amino acid uptake (25). The decreased transport was mediated primarily by increases in the apparent $K_m$ for the transporter. PGE$_2$ actually increased $V_{max}$ slightly. The decrease in transport induced by RA was also mediated primarily by increases in the apparent $K_m$. In contrast to PGE$_2$, the RA induced both an increase in $K_m$ and a decrease in $V_{max}$. These alterations in transporter function resulted in larger decreases in the rate of transport than that induced by PGE$_2$. The efflux studies indicate that this effect appears to be bidirectional (28). The decreases in the rate of transport was not the result of synthesis of a protein which either modifies or degrades the transporter because cycloheximide did not inhibit RA actions on amino acid uptake. In addition, RA did not function through PG release because the addition of indomethacin did not affect RA induced inhibition of proline transport.

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Retinoic Acid and Amino Acid Transport

Fig. 5. The effect of all-trans-RA on the uptake of [14C]AIB, [3H]leucine, and [14C]MeAIB. Quiescent fibroblast cultures were incubated in the presence or absence of all-trans-RA (10⁻⁶ M). A, the medium was removed and replaced with medium containing [14C]AIB at 1 μCi/0.1 mM or [3H]leucine at 1 μCi/0.5 mM and uptake was determined. Data are expressed as mean ± S.E.; n = 3. B, the medium was replaced with medium containing [14C]MeAIB and uptake was determined.

Fig. 6. The effect of all-trans-RA on efflux of [14C]AIB. The cells were incubated in the presence (closed circles) or absence (open circles) of all-trans-RA (10⁻⁶ M) for 24 h. The cells were loaded with [14C]AIB at 1 μCi/0.1 mM for 1 h and efflux was determined. The ratio of cellular [14C]AIB remaining after this incubation interval to the initial cellular [14C]AIB concentration, [AIB]₀/[AIB]ₚ, is plotted on the ordinate.

Fig. 7. The effect of cycloheximide and indomethacin on RA-induced decrease in [3H]proline uptake. Cultures were incubated in the presence or absence of all-trans-RA (10⁻⁶ M) or cycloheximide (CHX) at 5 μM or indomethacin (INDO) at 5 μM for 8 h. The medium was replaced with medium containing [3H]proline at 1 μCi/0.1 mM, and uptake was determined. The data are expressed as mean ± 1 S.E.; n = 3.

Fig. 8. Reciprocal velocity of AIB uptake plotted against reciprocal of AIB concentration. Cultures were maintained in the presence (closed circles) or absence (open circles) of RA (10⁻⁶ M) for 24 h. Uptake of AIB was determined at the indicated concentration of AIB (S, μM⁻¹). Velocity is expressed as nmol/min/mg of protein; n = 2. The V_max in control and RA-treated cultures were 1.98 and 1.66. The apparent K_m in control and RA-treated cultures were 2.14 and 6.80.

this time whether the decreases in amino acid transport result in alterations in the collagen mRNA levels. The RNA content of cells, however, is affected by amino acid concentration in the media (43) and specific mRNAs appear to be particularly sensitive to amino acid deprivation (44).

Our results suggests that RA may exert its affects on cellular function, in part, by modulating the activity of the Na⁺-dependent A transport system for amino acids. This does not appear to be a toxic effect because removal of the RA resulted in restitution of transporter function. Some studies have reported a functional association between the Na⁺,K⁺ pump and the A transport system (34). Our results indicate that the activity of the Na⁺,K⁺-ATPase is preserved in membrane preparations derived from RA-treated cultures.

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REFERENCES


