Hypoxia inhibits amino acid uptake in human lung fibroblasts

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Berk, John L., Christine A. Hatch, and Ronald H. Goldstein. Hypoxia inhibits amino acid uptake in human lung fibroblasts. J Appl Physiol 89: 1425–1431, 2000.—Hypoxia and amino acid deprivation downregulate expression of extracellular matrix genes in lung fibroblasts. We examined the effect of hypoxia on amino acid uptake and protein formation in human lung fibroblasts. Low O2 tension (0% O2) suppressed incorporation of [3H]proline into type I collagen without affecting [35S]methionine labeling of other proteins. Initial decreases in intracellular [3H]proline incorporation occurred after 2 h of exposure to 0% O2, with maximal suppression of intracellular [3H]proline levels at 6 h of treatment. Hypoxia significantly inhibited the uptake of radio-labeled proline, 2-aminoisobutyric acid (AIB), and 2-(methyl-amino)isobutyric acid (methyl-AIB) while inducing minor decreases in leucine transport. Neither cycloheximide nor indomethacin abrogated hypoxia-related suppression of methyl-AIB uptake. Efflux studies demonstrated that hypoxia inhibited methyl-AIB transport in a bidirectional fashion. The downregulation of amino acid transport was not due to a toxic effect; function recovered on return to standard O2 conditions. Kinetic analysis of AIB transport revealed a 10-fold increase in K0 accompanied by a small increase in maximal transport velocity among cells exposed to 0% O2. These data indicate that low O2 tension regulates the system A transporter by decreasing transporter substrate affinity.

ACUTE LUNG INJURY DISRUPTS vascular endothelial cell integrity and type 1 alveolar epithelial tight junctions, flooding terminal air spaces with protein-rich fluid and inflammatory cells (1, 31). Release of proteases from neutrophils and macrophages damages connective tissue elements within the interstitium, producing alveolar collapse and regions of hypoxia within the lung (18, 30, 31). Repair of protease-disrupted alveolar structures requires production of type I procollagen and tropoelastin molecules by interstitial lung fibroblasts (38). Frequently, extracellular matrix repair is incomplete, suggesting that low O2 tensions impair formation of type I collagen and tropoelastin by lung fibroblasts.

In the heart, kidney, and skin, hypoxia stimulates production of connective tissue elements by mesenchymal cells (2, 11, 23). Recently, however, we reported that low O2 tensions downregulate production of tropoelastin and α1(I) collagen molecules in interstitial lung fibroblasts by a complex interplay of transcriptional and posttranscriptional events (3). The exact mechanism by which hypoxia inhibits these genes remains undefined. Retinoic acid (RA), PGE2, and amino acid deprivation also downregulate α1(I) gene expression in cultured lung fibroblasts (12, 13, 26). All three perturbations decrease free intracellular pools of proline, a major constituent of collagen protein, before affecting α1(I) collagen mRNA levels (16, 25, 32). RA and PGE2 alter intracellular proline levels by suppressing its uptake into cultured lung fibroblasts (16, 25). Proline, a neutral aliphatic amino acid, is taken up predominantly by the A transport system, an Na+-dependent mechanism regulated by hormones, growth factors, cell volume changes, and nutrient depletion (15, 27, 29). The temporal sequence of events suggests that, like RA and PGE2, hypoxia may inhibit type I collagen gene expression in lung fibroblasts by decreasing proline uptake.

Precedent exists for regulation of amino acid transporters by low O2 tension. Hypoxia inhibits select amino acid transporters in pulmonary artery endothelial cells (PAEC) (5). Exposure of PAEC to 0% O2 for 4–20 h suppressed l-arginine uptake through the γ+ and B+ transports (5). Inhibition of amino acid uptake is not a uniform response to hypoxia. Indeed, prolonged hypoxia did not affect transport of L-citrulline or L-glutamine through the Na+-dependent ASC system into these cells (39). In rat cardiac myocytes, hypoxia stimulates glutamate through the XAG system (10). Whether hypoxia alters amino acid transport in lung fibroblasts is unknown.

We examined the effect of hypoxia on collagen formation and proline uptake through the A system amino acid transporter in human lung fibroblasts. Hypoxia decreased type I collagen production while it downregulated the activity of the A transport system.

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METHODS

Cells and cell culture. Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in DMEM with 0.37 g sodium bicarbonate/dl, 10% fetal bovine serum, 100 U penicillin/ml, 10 μg streptomycin/ml, and 0.1 mM nonessential amino acids. The cells were grown in 35-mm dishes (Falcon, Oxford, CA) and maintained in a humidified 5% CO₂-95% air incubator at 37°C. When confluent, cells were made quiescent 1 day before stimulation by reducing the media serum content to 0.4%. During experiments, cells were incubated at 37°C in 5% CO₂-95% air or in a humidified sealed chamber (Billups-Rothenburg, Del Mar, CA) gassed with 0, 3, or 10% O₂ containing 5% CO₂-balance N₂ mixtures (Medical-Technical Gases, Medford, MA). When incubated in a 0% O₂ environment, O₂ tension in the culture media falls from 139 ± 11 Torr to a steady-state 20–32 Torr over the initial 12 h (24). All experiments were performed in serum-free media. Cell number was determined by triplicate cell counts with an electronic particle counter (Coulter Counter ZM).

Reagents. L-[3H]proline (102 Ci/mmol), L-[4,5,3H]leucine (46 Ci/mmol), [35S]methionine (1,175 Ci/mmol), 2-(methylamino)-1-[14C]isobutyric acid (methyl-AIB, 57 mCi/mmol), and 2-amino-1-[14C]isobutyric acid (58 mCi/mmol) were purchased from Dupont-New England Nuclear (Boston, MA).

PAGE. Confluent quiescent fibroblasts were labeled with [3H]proline (5 μCi/ml) and ascorbate (50 μg/ml) in the presence of different concentrations of O₂. After treatment, medium was collected, with the addition of protease inhibitors yielding final concentrations of 10⁻⁴ M phenylmethylsulfonyl fluoride, 10⁻⁴ M hydroxymercuribenzoate, and 2 × 10⁻³ M EDTA. Samples containing equal volumes of media from equal numbers of cells were dialyzed against H₂O at 4°C, lyophilized, and digested with pepsin on 4°C overnight. After repeat dialysis and lyophilization, aliquots were separated by PAGE on a 7.5% gel (28). Autoradiography was performed according to the methods of Bonner and Laskey (6). The identity of the collagen bands was confirmed by comigration with pepsin-treated radiolabeled type I collagen and collagenase sensitivity (14, 25). To examine protein synthesis, cell cultures were exposed to low O₂ tension with substitution of 0% O₂ did not significantly affect free intracellular [3H]proline levels in the TCA-soluble and TCA-insoluble fractions were assessed by liquid scintilligraphy (Table 1). The radiolabeled free intracellular proline pools (TCA-soluble fractions) were unaffected by 0% O₂ at 2 h, decreasing to 46% of untreated controls after 6 h of treatment (P < 0.02). In contrast, 2 h of 0% O₂ suppressed levels of [3H]proline-labeled proteins (TCA-insoluble fractions) to 69% of control values, a trend that did not reach statistical significance. By 6 h of 0% O₂, radiolabeled proteins fell to 62% of levels present in untreated cultures (P < 0.05). Exposure to low O₂ tension for 16 h had little additional effect on [3H]proline-labeled proteins. Although brief (2 h) exposure to 0% O₂ did not significantly affect free intracellular [3H]proline pools or [3H]proline-labeled protein levels, extended exposure did suppress TCA-soluble and TCA-insoluble [3H]proline pools. These data suggest that low O₂ tensions inhibit proline-rich protein formation, perhaps by limiting free intracellular proline pools.

RESULTS

To examine the effect of low O₂ tensions on type I collagen expression in the extravascular lung, we exposed human lung interstitial fibroblasts (IMR-90) to various concentrations of O₂ for 30 h in the presence of [3H]proline. Culture media were digested with pepsin and electrophoretically resolved (Fig. 1). By densitometric analysis, 0% O₂ decreased the α1(I) collagen signal to 25% of 21% O₂ control values; 3% O₂ induced minor inhibition (78% of controls), whereas 10% O₂ did not suppress steady-state α1(I) levels. Low O₂ tensions did not affect cell morphology or adherent cell counts in cultures treated in parallel fashion (data not shown).

Proline is highly represented in the primary structure of type I collagen. To determine the effect of low O₂ tension on the fate of intracellular proline, lung fibroblasts were untreated or cultured in 0% O₂ for various periods of time in the presence of radiolabeled proline. After treatment, cells were extracted with 10% TCA; [3H]proline levels in the TCA-soluble and TCA-insoluble fractions were assessed by liquid scintilligraphy (Table 1). The radiolabeled free intracellular proline pools (TCA-soluble fractions) were unaffected by 0% O₂ at 2 h, decreasing to 46% of untreated controls after 6 h of treatment (P < 0.02). In contrast, 2 h of 0% O₂ suppressed levels of [3H]proline-labeled proteins (TCA-insoluble fractions) to 69% of control values, a trend that did not reach statistical significance. By 6 h of 0% O₂, radiolabeled proteins fell to 62% of levels present in untreated cultures (P < 0.05). Exposure to low O₂ tension for 16 h had little additional effect on [3H]proline-labeled proteins. Although brief (2 h) exposure to 0% O₂ did not significantly affect free intracellular [3H]proline pools or [3H]proline-labeled protein levels, extended exposure did suppress TCA-soluble and TCA-insoluble [3H]proline pools. These data suggest that low O₂ tensions inhibit proline-rich protein formation, perhaps by limiting free intracellular proline pools.

To determine whether hypoxia altered the incorporation of other amino acids into proteins, cells were exposed to 21% or 0% O₂ in the presence of [35S]methionine (Fig. 2). Low O₂ tensions did not alter the pattern or relative intensity of proteins labeled by [35S]methi-
online, indicating that hypoxia did not suppress protein formation in a nonspecific fashion.

The decrease in intracellular radiolabeled proline levels suggested that hypoxia might inhibit proline uptake by lung fibroblasts. When exposed to 0% O2 for 6 h, [3H]proline uptake by IMR-90 fell to 62% of untreated controls within 10 min of introduction of the radiolabeled amino acid (Fig. 3A). This transport inhibition was sustained over the 20-min labeling period. Six hours of hypoxia also suppressed rapid incorporation of [3H]proline into proteins (Fig. 3B), supporting results from earlier steady-state radiolabeling experiments (Table 1). Proline uptake is principally mediated by the Na\(^{+}\)-dependent A transport system in lung fibroblasts (16, 25). We used radiolabeled AIB ([14C]AIB), an amino acid analog that is not degraded or incorporated into proteins (29), to examine the effect of hypoxia on the A transport system. As noted in steady-state measures of intracellular proline, 2 h of 0% O\(_2\) did not affect AIB uptake; however, more prolonged treatment suppressed AIB transport in a dose relationship. Specifically, 6 h of 0% O\(_2\) decreased AIB uptake to 74 ± 14% (mean ± SE, n = 3) of 21% O\(_2\) values, whereas 16 h of treatment inhibited uptake to 45 ± 6% (n = 3, P < 0.03) of controls (Fig. 4). To establish whether hypoxia selectively altered function of the A transport system, we assessed the effect of 0% O\(_2\) on uptake of radiolabeled leucine, a polar amino acid taken up by the Na\(^{+}\)-independent L transport system (29, 35). In contrast to A system transport, 0% O\(_2\) induced minor suppression of [3H]leucine uptake at 6 h (82 ± 7% of control) and 16 h (75 ± 4%, mean ± SE, n = 3, not significant; Fig. 4). These data indicated that hypoxia predominantly affected uptake of amino acids through the A transport system in lung fibroblasts.

We examined the reversibility of hypoxic suppression of [14C]AIB transport. After IMR-90 cells were exposed to 21% or 0% O\(_2\) for 2–24 h, AIB uptake was assessed (Fig. 5). Additional cultures treated in parallel.

**Table 1. Effect of hypoxia on intracellular [3H]proline pool**

<table>
<thead>
<tr>
<th>Time, h</th>
<th>TCA-Soluble [3H]proline</th>
<th>TCA-Insoluble [3H]proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4,722 ± 773</td>
<td>4,470 ± 241</td>
</tr>
<tr>
<td>6</td>
<td>6,394 ± 951</td>
<td>2,968 ± 357</td>
</tr>
<tr>
<td>16</td>
<td>5,743 ± 245</td>
<td>4,472 ± 460</td>
</tr>
<tr>
<td></td>
<td>21% O(_2)</td>
<td>0% O(_2)</td>
</tr>
<tr>
<td></td>
<td>420 ± 146</td>
<td>291 ± 85</td>
</tr>
<tr>
<td></td>
<td>983 ± 108</td>
<td>608 ± 38</td>
</tr>
<tr>
<td></td>
<td>1,831 ± 63</td>
<td>1,066 ± 182</td>
</tr>
</tbody>
</table>

Values are means ± SE in counts per minute per sample; n = 3. Confluent quiescent IMR-90 cells were labeled with [3H]proline during treatment with 21% or 0% O\(_2\) for 2, 6, or 16 h. After treatment, cells were washed and collected by scraping. Intact proteins were precipitated by 10% TCA. Radioactivity in the supernatants and TCA pellets was determined by liquid scintilligraphy.

**Fig. 3.** Effect of hypoxia on uptake (A) and incorporation (B) of [3H]proline into cellular proteins in quiescent lung fibroblasts. Fibroblasts were treated with 21% or 0% O\(_2\) for 6 h. After treatment, [3H]proline uptake was determined. Cultures were washed and refed media including [3H]proline (1 μCi/0.1 mM). At specific time intervals, intact proteins in the cell layer were precipitated with cold TCA. Radioactivity of the supernatants and pellets was determined by liquid scintilligraphy. A: levels of radioactivity in supernatants of TCA-precipitated samples (amino acid uptake). B: counts in pellets of precipitated samples (protein incorporation). Values are means ± SE; n = 3. cpm, Counts/min.

**Fig. 2.** Effect of low O\(_2\) tensions on synthesis of proteins by cultured lung fibroblasts. IMR-90 cells were treated with 0, 3, 10, or 21% O\(_2\) for 24 h in the presence of [35S]methionine. Media were harvested. Aliquots from each condition containing equal counts were separated by electrophoresis on 7.5% polyacrylamide gel. A representative autoradiograph is presented.
lel with 21% or 0% O₂ were fed fresh media after stimulation and placed in 21% O₂ to recover for 30 h. Hypoxia-related inhibition of AIB uptake was completely reversible after 2, 6, and 24 h of exposure, indicating that this was not a toxic effect. Previously, we showed that PGE₂ downregulates A system transport in cultured lung fibroblasts (16). Additionally, under certain conditions, regulation of A system transport requires ongoing protein formation (40). To determine the role of protein synthesis and prostaglandins on hypoxia-induced suppression of methyl-AIB uptake, cells were treated with 0% O₂ in the presence and absence of cycloheximide or indomethacin. Cycloheximide at 5 μM inhibits 85% of protein synthesis in human lung fibroblasts (17). As reported in these cells (16), cycloheximide alone decreased methyl-AIB uptake (Fig. 6), suggesting the constitutive expression of an activating protein. When combined with 0% O₂, however, cycloheximide did not block hypoxia-induced downregulation of AIB uptake. Indomethacin, a cyclooxygenase inhibitor, did not affect methyl-AIB uptake in 21% O₂, nor did it abrogate suppression of methyl-AIB uptake by hypoxia (Fig. 6). The effects of low O₂ tension were not limited to uptake capacities of amino acid transport. Exposure to 0% O₂ induced bidirectional suppression of methyl-AIB transport, decreasing efflux by 19% (Fig. 7).

To more closely define the mechanism of hypoxia-induced inhibition of A system transport, we performed kinetic studies. Prior experiments (Fig. 5) revealed significant decreases in AIB uptake after 6 h of hypoxia. At 6 h of treatment, hypoxia effected a 2-fold increase in maximal transport velocity (Vₘₐₓ) and a 10-fold increase in Kₘ (Fig. 8). These data suggest that decreases in A system transporter-ligand affinity explain the downregulation of AIB uptake by hypoxia, overwhelming modest upregulation of transporter cell surface expression.

**DISCUSSION**

Exposure to low O₂ tension for 6 h decreased system A amino acid transport of proline, AIB, and methyl-AIB in human lung fibroblasts. Returning cell cultures to 21% O₂ completely restored system A function, suggesting that changes in amino acid transport induced by up to 24 h of treatment with 0% O₂ were not attributable to toxic events. Hypoxia appeared to preferentially downregulate amino acid transport through the A system, as only minor decreases occurred in L system uptake. Moreover, 0% O₂ inhibited [³H]proline uptake and radiolabeling of type I collagen without affecting the expression of methionine-rich proteins.

Hypoxia inhibited AIB and methyl-AIB uptake by reducing ligand affinity of the transporter 10-fold,
HYPOXIA INHIBITS AMINO ACID UPTAKE

Hypoxia does not suppress transport of all amino acids. Glutamate, an acidic amino acid, is transported principally in an electrogenic fashion by system X_AG (19, 29). Despite depleting intracellular l-glutamate levels, 30 min of treatment with 0% O_2 stimulated l-glutamate uptake in rat cardiac myocytes by increasing X_AG \( V_{\text{max}} \) 1.6-fold (10). Hypoxia also induced uptake through low-affinity, high-capacity systems, including ASC, X_C, and L (10).

We employed AIB and methyl-AIB to examine the effect of hypoxia on system A function. In Chinese hamster ovary cells, AIB and proline have similar transporter profiles (~70% uptake by system A), with minor transport occurring through system L and ASC, respectively (34). Methylation of AIB (methyl-AIB) maximizes its transport through system A. In lung fibroblasts, hypoxia inhibited methyl-AIB uptake to a greater degree (~95%) than it did AIB uptake (~60%). System L transporters, the minor component of AIB uptake, demonstrated significantly less hypoxia-induced suppression. Therefore, data employing AIB substrate appear to underestimate the inhibitory effects of hypoxia on system A function in lung fibroblasts.

Intracellular proline levels are determined by uptake of extracellular proline stores, rates of intracellular protein degradation, and, in rat xiphoid cartilage, endogenous synthesis from L-ornithine (36). In human lung fibroblasts, however, exogenous proline stores appear principally responsible for sustaining free intracellular proline pools, as demonstrated by experiments limiting extracellular proline availability. When cultured in amino acid-deficient media or stimulated by PGE_2, total free intracellular proline levels in lung fibroblasts decreased significantly (27). PGE_2 alone suppressed system A transport and intracellular levels of proline without altering the specific activity of free intracellular proline pools used to charge tRNA (16, 25). Were intracellular proline pools sustained by re-

![Fig. 7. Effect of hypoxia on methyl-AIB efflux. Confluent quiescent fibroblasts were treated with 0% or 21% O_2 for 6 h, washed, and loaded with [14C]methyl-AIB (1 \( \mu \)M/0.1 mM) for 1 h. Efflux was determined from 0 to 90 s. Ordinate, ratio of intracellular [14C]methyl-AIB present after efflux incubation to intracellular [14C]methyl-AIB levels measured immediately after cell loading ([14C]Me-AIBt/[14C]Me-AIB0).](Image 67x551)

![Fig. 8. Lineweaver-Burk plot: reciprocal velocity of AIB uptake plotted against reciprocal AIB concentration. IMR-90 cells were exposed to 21% or 0% O_2 for 6 h. Uptake of [14C]AIB was assessed at the indicated substrate concentrations. Velocity (V) is expressed as nmol·min⁻¹·mg protein⁻¹; n = 2. \( V_{\text{max}} \) in cultures treated with 21% and 0% O_2 were 0.93 and 2.29, respectively; apparent \( K_m \) in cells treated with 21% and 0% O_2 were 0.46 and 4.63 mM, respectively.](Image 537x293)
cycled proline or by endogenous proline formation, specific activity of intracellular proline pools would decline over time. The stability of the specific activity of intracellular proline pools supports the importance of exogenous proline transport in replenishing other proline pools. We did not determine the specific activity of ^3H(proline-labeled proteins; however, the constant ratio of radiolabeled proteins in the hypoxic and normoxic groups suggested that low O_2 tension may reduce intracellular proline pools without promoting compensatory protein degradation or endogenous proline synthesis. Further studies are needed to establish the effect of hypoxia on specific proline pools in lung fibroblasts.

Exposure to low O_2 tension for 12–24 h downregulates extracellular matrix gene mRNA levels in cultured lung fibroblasts (3). In contrast, hypoxia inhibits proline uptake through the A system transporter after only 2–6 h of treatment of IMR-90 cells. This temporal relationship suggests that hypoxia may suppress type I collagen gene expression by limiting the availability of vital amino acids such as proline. Previously, we have shown that amino acid deprivation of human lung fibroblasts decreases intracellular levels of proline, glycine, alanine, and leucine followed by selective inhibition of α1(I) mRNA expression (27). In rat hepatoma cells, amino acid deprivation suppresses histone H2, superoxide dismutase, and glyceraldehyde 3-phosphate dehydrogenase mRNA levels (33). Inhibition of gene expression is not a universal response to starvation, however. Asparagine synthase mRNA levels increased in baby hamster kidney cells cultured under amino acid-free conditions (20, 22). Further studies are needed to determine whether hypoxia and amino acid deprivation downregulate α1(I) mRNA by the same mechanisms.

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