Regulation of ornithine decarboxylase and polyamine import by hypoxia in pulmonary artery endothelial cells

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In the lung, chronic hypoxia causes extension of smooth muscle (or smooth muscle-like) cells into normally nonmuscular pulmonary arteries, hypertrophy of smooth muscle cells in conduit pulmonary arteries (23–25), and alterations in the deposition of extracellular matrix proteins (11, 12, 22, 35). These anatomic changes, probably acting in concert with elevated vasomotor tone (28), lead to sustained increases in pulmonary vascular resistance and right ventricular hypertrophy.

The polyamines, putrescine, spermidine, and spermine, are a family of low-molecular-weight organic cations that contribute to many aspects of signal transduction. It has been recognized for many years that precise adjustments in polyamine pools are required for cell growth and differentiation (20). More recent data indicate that the polyamines exhibit substantial antioxidant activity (14, 27), regulate certain ion channels and receptors (6, 7, 17), and modulate a number of mitochondrial functions (21, 30). Given the apparent central role of polyamines in regulating cell proliferative and functional states, their importance in governing responses of pulmonary vascular cells in hypoxia seems likely. In this regard, previous reports have shown that lung polyamine contents are elevated in hypoxia (29) and that pharmacological inhibition of this increase attenuates hypoxic pulmonary vascular remodeling and hypertension (1).

Mechanisms regulating pulmonary vascular cell polyamine contents in hypoxia are incompletely understood and may differ from those operative in nonpulmonary cells. For example, whereas in hypoxic brain activity of the initial and generally rate-limiting enzyme in polyamine synthesis ornithine decarboxylase (ODC) is elevated (18, 19), in hypoxic lung and cultured pulmonary arterial effector cells, ODC activity is decreased (3, 15, 16, 33). Instead, lung cell polyamine uptake is augmented in hypoxia (3, 16, 33). Thus the most likely basis for the increased lung polyamines required for chronic hypoxia-induced pulmonary hypertension is induction of transmembrane polyamine transport in pulmonary arterial endothelial and smooth muscle cells. The mechanism by which hypoxia depresses ODC and stimulates polyamine uptake has not been examined.

It has been demonstrated repeatedly that inhibition of ODC by the site-specific “suicide” inhibitor α-difluoromethylornithine (DFMO) causes compensatory induction of polyamine import, presumably as a means of forestalling potentially toxic polyamine depletion (31, 32). It is thus reasonable to postulate that a similar mechanism could be operative in hypoxic lung vascular cells; hypoxia could cause a primary decrease in ODC activity, pulmonary endothelial cells; pulmonary hypertension; polyamines.
activity that, in turn, could activate transmembrane polyamine import. A potential mediator of the hypoxia-induced reduction of ODC activity is antizyme, a family of 18- to 30-kDa proteins that promotes the 26S proteasome-dependent degradation of ODC and, at least for some forms of antizyme, also inhibits transmembrane polyamine transport (9, 10). Consequently, because hypoxia reduces ODC activity but enhances polyamine transport, involvement of the antizyme pathway of polyamine regulation in the hypoxic response is unclear. Accordingly, the present study determined in pulmonary artery endothelial cells (PAEC) whether hypoxia decreased ODC activity, explored whether enhanced ODC degradation mediated by antizyme could be responsible for the decrease, and tested the idea that decreased ODC activity was a proximate cause of the increase in polyamine transport.

METHODS

Rat main PAEC cultures and hypoxic exposure. Rat main PAEC were isolated and cultured as described previously (3, 4). In brief, main pulmonary arteries were isolated from 250- to 300-g Sprague-Dawley rats killed with an overdose of nembutal. Isolated arteries were opened, and the intimal lining was scraped carefully with a scalpel. The harvested cells were then placed in flasks (Corning, Corning, NY) containing F-12 Nutrient Mixture and DMEM mixture (1:1) supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO-BRL, Grand Island, NY). Culture medium was changed one time per week, and, after reaching confluence, the cells were harvested using a 0.05% solution of trypsin (GIBCO-BRL). The endothelial cell phenotype, confirmed by acetylated lipopolysaccharide uptake, factor VIII-RAg immunostaining, and the lack of immunostaining with smooth muscle cell a-actin antibodies (Sigma, St. Louis, MO), persisted for at least 15 passages.

In all experiments, PAEC were cultured on 100-mm petri plates in Plexiglas, environmentally controlled chambers. Normoxic cells were exposed to an atmosphere of 95% air-5% CO2, and “hypoxic” cells were exposed to 2% oxygen-5% CO2-balance N2.

ODC activity and mRNA analyses. ODC activity in PAEC was evaluated as previously described (15). ODC activity, quantified as the amount of [32P]CTP-labeled single-stranded DNA probe overnight at 37°C, was normalized to cellular protein content, as determined by the Bradford assay.

To determine whether hypoxia altered ODC mRNA, 20 μg total RNA were ultravioletly cross-linked to a Nytran membrane (Schleicher and Schuell, Keene, NH) and hybridized to [32P]CTP-labeled single-stranded DNA probe overnight at 55°C. Integrated density values for the intensity of hybridization were obtained using a Molecular Image Storage Phosphor Imaging system (Bio-Rad, Hercules, CA). Values were normalized to the extent of cyclophillin mRNA expression determined using a similar protocol.

Western analysis of ODC antizyme. Cells were seeded in six-well plastic plates at 5 × 105 cells/well and, when confluence was attained, exposed to hypoxia or lactacystin for the indicated durations. Cells were then washed two times with PBS and lysed in 3% SDS electrophoresis buffer, and 120 μg protein were applied to an SDS-12% polyacrylamide gel. After separation, samples were transferred to nitrocellulose filters (Bio-Rad). Membranes blocked in 5% nonfat dried milk in PBS with 0.1% Tween 20 (PBS-T) were incubated over-night at 4°C with primary, polyclonal antibody recognizing both antizyme-1 and antizyme-2 (generously provided by Dr. J. L. Mitchell), diluted 1:1,000 in PBS-T with 0.5% BSA. Before the membrane was applied, the diluted antibody was filtered through a 0.45-μm nylon filter (Corning). After being washed, the membranes were incubated with 1,750 diluted horseradish peroxidase-conjugated goat antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and then were revealed by chemiluminescence with the SuperSignal West Pico kit (Pierce, Rockford, IL).

RESULTS

In intact rat lung (33) and cultured bovine pulmonary artery smooth muscle cells (2, 16), hypoxia decreases ODC activity, but this has not yet been determined in rat cultured main PAEC. Accordingly, in initial studies, we verified in rat lung endothelial cells that hypoxia caused time-dependent reductions in ODC activity, which, as shown in Fig. 1, resulted in nearly complete inhibition within 24 h of hypoxic exposure.

Two plausible mechanisms whereby hypoxia could decrease ODC activity include transcriptional down-regulation, which appears to contribute to the decreased ODC activity noted in pulmonary artery smooth muscle cells (15), and induction of antizyme.
which promotes ODC degradation by a 26S proteasome via a ubiquitin-independent pathway (9, 10). To address involvement of these two mechanisms in hypoxic endothelial cells, we measured ODC mRNA by Northern slot-blot analysis and antizyme by Western analysis. As depicted by the representative autoradiogram shown in Fig. 2, hypoxia promotes a time-dependent decrease in ODC mRNA. When hybridization intensities were quantified by densitometry and normalized to expression of the housekeeping gene cyclophilin, we found that ODC mRNA abundance was reduced compared with normoxic controls after 12 and 24 h of hypoxic exposure. In terms of the antizyme regulatory pathway, a representative autoradiogram of a Western analysis for the 24-kDa antizyme abundance is shown in Fig. 3 and suggests that antizyme did not change during hypoxia exposure. When band intensity was quantified by densitometry, normalized to the abundance of actin (Western analysis not shown), and expressed as a percentage of control for at least four experiments, we found that up to 24 h of hypoxic exposure failed to elevate abundance of the 24-kDa antizyme isoform. Another putative antizyme isoform of ~30 kDa was present at the lower limit of detection and was also not altered by hypoxia (data not shown). As positive controls, antizyme abundance was determined in lactacystin-treated endothelial cells and in ODC-overexpressing cells, both of which exhibit substantial increases in ODC activity (see below). Also as shown in Fig. 3, each of these interventions caused substantial increases in antizyme.

We next used two different strategies to determine if suppression of ODC degradation prevented the hypoxia-induced reduction in ODC activity. In the first, PAEC were treated with the proteasome inhibitor lactacystin (5 \mu M) for 12 h. Control and lactacystin-treated PAEC were then cultured for an additional 24 h under normoxic or hypoxic conditions after which ODC activity was measured. As shown in Fig. 4A, ODC activities in cells treated with lactacystin were increased substantially compared with normoxic controls, as would be predicted if proteasome-dependent ODC degradation were suppressed. Hypoxia, also as expected, reduced ODC activity, whereas in cells treated with lactacystin, ODC activity did not decrease below the elevated baseline established by lactacystin treatment. The second approach used rat lung endothelial cells stably transfected with a truncated, mutant ODC resistant to proteasomal degradation. Figure 4B shows that, similar to its impact in wild-type cells, hypoxia decreases ODC activity in neo-transfected PAEC, which are the control for the truncated ODC mutant. The effect of hypoxia on ODC activity in the overexpressing truncated mutant is depicted in Fig. 4C. Note that, in normoxic cells, baseline ODC activity is considerably elevated compared with the neo transfecants (Fig. 4A), as would be expected, since the
truncated mutant is resistant to degradation. However, hypoxia fails to reduce ODC activity in PAEC overexpressing the truncated mutant.

Hypoxia increases polyamine import in pulmonary artery smooth muscle and endothelial cells and in intact lung (2, 3, 33). Because pharmacological inhibition of ODC activity with DFMO causes large increases in polyamine import in virtually all cell types so far studied (31, 32) and because hypoxia decreases ODC activity, it was reasonable to postulate that a proximate stimulus for increased polyamine import in hypoxic PAEC could be the reduction in ODC activity. To address this issue, we determined if the two strategies noted above, lactacystin and the truncated ODC mu-

Fig. 3. A: Western analysis of ODC antizyme in normoxic pulmonary artery endothelial cells and in cells cultured for up to 24 h under hypoxic conditions. As positive controls for increasing antizyme, antizyme abundance was determined in cells treated with lactacystin (5 μM; LACT) for 24 h and in cells transfected with a truncated ODC mutant resistant to proteasome-mediated degradation (ODC). Note lack of increased antizyme content in hypoxic cells relative to control and marked elevation in lactacystin-treated and ODC-overexpressing cells. B: antizyme abundance was quantified by densitometry in cells cultured up to 24 h in hypoxia, normalized to the abundance of actin for 4 experiments, and expressed as a percentage of normoxic controls. There were no differences in antizyme abundance from control at any duration of hypoxia exposure.

Fig. 4. A: ODC activity in pulmonary artery endothelial cells cultured in normoxia in the absence and presence of 5 μM lactacystin for 24 h or for 24 h in hypoxia in the absence (HYP) and presence of lactacystin (H + L). B and C: ODC activities in neo-transfected pulmonary artery endothelial cells and in pulmonary arterial endothelial cells transfected with a truncated, degradation-resistant ODC mutant, respectively. Cells were cultured in either normoxia or hypoxia for 24 h after which ODC activity was measured. Bars reflect means ± SE of at least 4 determinations. *Significantly different from normoxia at P < 0.05.
tant, would prevent the hypoxic induction of polyamine transport. Uptakes of 3 μM [14C]putrescine, [14C]spermidine, and [14C]spermine were determined after 30 min of incubation with the radiolabeled polyamine using the same experimental design described above to examine the impact of lactacystin and the truncated ODC mutant on the hypoxic reduction in ODC activity. As expected from our earlier work (2, 3), Fig. 5, A and B, shows that, compared with cells cultured under normoxic conditions, hypoxia increased the uptake of all three 14C-labeled polyamines in both wild-type cells and the neo transfectants serving as controls for the truncated ODC mutants. Surprisingly, in view of their ability to increase antizyme abundance, polyamine transport in normoxic cells was markedly elevated by treatment with lactacystin and in cells expressing the degradation-resistant ODC mutant. Hypoxia was incapable of promoting further increases in any 14C polyamine uptake in rat PAEC treated with lactacystin or expressing the truncated form of ODC.

It is possible that the failure of hypoxia to elevate polyamine import in lactacystin-treated or mutant ODC-overexpressing endothelial cells was related to their elevated baseline polyamine transport rates; polyamine transport could have been maximally activated by these treatments, and thus no further increase with hypoxia was attainable. To determine if polyamine import was maximally activated by the strategies for impairment of ODC degradation, we treated cells with lactacystin for 24 h, and, for the last 12 h of incubation, some cultures were treated with the ODC inhibitor DFMO at a final concentration of 5 μM. DFMO has been shown to repeatedly elevate the polyamine transport rate in virtually all cells studied so far (31, 32). Data presented in Fig. 6 indicate that DFMO treatment increased polyamine transport to approximately the same extent as lactacystin and, importantly, that DFMO promoted significant increases beyond the elevated baseline established by lactacystin for [14C]putrescine and [14C]spermidine and tended to do so for [14C]spermine. The inability of hypoxia to cause increased polyamine import in lactacystin-treated endothelial cells shown in Fig. 5 can thus not be ascribed to the fact that the transport pathway was maximally activated.

DISCUSSION

This work extends previous findings in intact rat lung (33) and cultured bovine pulmonary artery smooth muscle (15, 16) to cultured rat main PAEC by showing that hypoxia depresses ODC activity. In addition, it confirms our previous observations in pulmonary artery explant preparations that hypoxia elevates polyamine import in main PAEC (3). The more interesting aspects of the current study, pertaining to the relationship between the hypoxic reduction in ODC activity and increases in polyamine transport and the potential involvement of antizyme in these events, are discussed below.

Results of the present study show that two disparate interventions known to suppress ODC degradation, lactacystin treatment and use of degradation-resistant

ODC mutant, both prevented the hypoxic reduction in ODC activity. Interestingly, the abundance of antizyme, which accelerates ODC degradation (9, 10), was not elevated in hypoxic PAEC at any time during the 24-h hypoxic exposure. The antibody used in the present studies detects the best-recognized and most prevalent member of the antizyme family, the 24-kDa isoform (26), which appears to be the dominant form present in PAEC. Collectively, these findings suggest that ODC degradation in hypoxic endothelial cells occurs without the requirement for increased antizyme abundance. Such a lack of involvement of antizyme in the diminished ODC activity in hypoxia joins a growing list of conditions in which a role for antizyme cannot be unambiguously determined, including the inhibitory effect of dexamethasone on newborn rat lung ODC (5), the reduction in ODC activity caused by inhibition of S-adenosylmethionine decarboxylase in the IEC-6 intestinal epithelial cell line (36), and ODC inhibition evoked by agmatine in rat PAECs (4).

If antizyme is not involved in the hypoxic reduction in ODC activity, then what is a probable mechanism of this response? We previously showed in bovine pulmonary artery smooth muscle cells that hypoxia was associated with reductions in ODC mRNA that were temporally related to the diminution in ODC activity (15). The hypoxia-mediated reduction in ODC mRNA could not be ascribed to a decrease in ODC mRNA stability, thus suggesting transcriptional downregulation of the enzyme. In the present study, we found that hypoxia also reduced the abundance of ODC mRNA in PAEC. The reduction in ODC mRNA tended to lag behind the decrease in ODC activity and did not attain statistical significance until 12 and 24 h of hypoxic exposure. This finding, when considered in light of observations that inhibition of proteasome-mediated ODC degradation with lactacystin and the truncated ODC mutant preserved ODC activity in hypoxia and that antizyme abundance did not increase, suggests that the hypoxia-induced suppression of ODC activity is linked to a combination of impaired gene expression and constitutive, antizyme-independent degradation of ODC protein.

As positive controls for the effect of hypoxia on antizyme abundance, we also determined antizyme in lactacystin-treated cells and in the overexpressing ODC mutants. Because of the large increases in ODC activity in these two treatment groups, it was not surprising that antizyme abundance would be increased (9, 10). What was unexpected, however, was that the large increase in antizyme associated with these two strategies was not accompanied by inhibition of polyamine uptake. On the contrary, polyamine import was enhanced markedly in both lactacystin-treated and the mutant ODC-overexpressing endothelial cells. In the case of lactacystin, one explanation for the failure of elevated antizyme to downregulate polyamine transport is that, when ODC degradation is suppressed by the proteasome inhibitor, antizyme remains bound to ODC and is therefore unavailable to inhibit the transporter. Such an argument cannot be advanced for the truncated mutant, since the segment of ODC to which antizyme binds is deleted. At this point, it seems most reasonable to suggest that the lack of close association between antizyme abundance and polyamine import in PAEC relates to the fact that the role of antizyme in this cell population is different from the others studied so far.

When the hypoxia-induced reduction in ODC activity was prevented by lactacystin or in the mutant ODC overexpressors, the expected increase in polyamine import did not occur. As noted above, both interventions to suppress ODC degradation were associated with large increases in the baseline polyamine transport, and it is thus possible that the failure of hypoxia to promote a further increase was related to the fact that the transport system was activated maximally. This does not seem to be the case, however, because the ODC inhibitor DFMO, which is a widely recognized stimulus for increased polyamine import (31, 32), retained its ability to activate the transport pathway beyond the elevated baseline associated with lactacystin treatment. It also should be considered that hypoxia failed to increase transport in the lactacystin-treated or mutant ODC-overexpressing endothelial cells because the elevated antizyme abundance inhibited further increases. This, too, seems unlikely in light of the fact that both of these interventions were linked to large increases in the baseline transport rate. In light of these considerations, the most reasonable conclusion is that the hypoxia-induced increase in polyamine transport is functionally related to the reduction in ODC activity.

In summary, the present study shows that hypoxia decreases ODC activity in rat PAEC as it does in other lung vascular cell populations. The decrease is not associated with elevated antizyme abundance but can be prevented by interventions inhibiting the proteasome-mediated degradation of the enzyme, thus suggesting that constitutive, antizyme-independent ODC degradation is important for the reduction in ODC activity. Finally, because the hypoxia-induced increase in polyamine transport does not occur when ODC activity is preserved, it seems most likely that these reciprocal changes in polyamine regulatory pathways are functionally linked. In retrospect, it is perhaps not surprising that a pathway independent of antizyme is involved with the regulatory effects of hypoxia on ODC activity and polyamine transport. Although the known isoforms of antizyme inhibit ODC and attendant polyamine synthesis and transmembrane polyamine transport and thus act to diminish polyamine contents in accord with the cellular need, hypoxia has divergent effects on ODC and polyamine transport; i.e., it inhibits ODC and augments transport. Moreover, lung vascular cells physiologically adapt to hypoxia, as revealed by alterations in matrix protein synthesis and deposition and growth factor expression, etc. (11, 12, 22, 34, 37), and such events require elevations in cellular polyamines (20). Thus, despite the reduction in ODC activity, the need for polyamines is evident. Understanding this apparently unusual regulatory process will require additional study.
REFERENCES


