Endothelial Cell Tolerance to Hypoxia

Potential Role of Purine Nucleotide Phosphates

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Abstract

The ability of cells to tolerate hypoxia is critical to their survival, but varies greatly among different cell types. Despite alterations in many cellular responses during hypoxic exposure, pulmonary arterial endothelial cells (PAEC) retain their viability and cellular integrity. Under similar experimental conditions, other cell types, exemplified by renal tubular epithelial cells, are extremely hypoxia sensitive and are rapidly and irreversibly damaged. To investigate potential mechanisms by which PAEC maintain cellular and functional integrity under these conditions, we compared the turnover of adenine and guanine nucleotides in hypoxia tolerant PAEC and in hypoxia-sensitive renal tubular endothelial cells under various hypoxic conditions. Under several different hypoxic conditions, hypoxia-tolerant PAEC maintained or actually increased ATP levels and the percentage of these nucleotides found in the high energy phosphates, ATP and GTP. In contrast, in hypoxia-sensitive renal tubular endothelial cells, the same high energy phosphates were rapidly depleted. Yet, in both cell types, there were minor alterations in the uptake of the precusor nucleotide and its incorporation into the appropriate purine nucleotide phosphates and marked decreases in ATPase and GTPase activity. This maintenance of high energy phosphates in hypoxic PAEC suggests that there exists tight regulation of ATP and GTP turnover in these cells and that preservation of these nucleotides may contribute to the tolerance of PAEC to acute and chronic hypoxia. (*J. Clin. Invest.* 1995. 95:738–744.) Key words: anoxia • adenosine triphosphate • guanosine triphosphate • cellular hypoxia • endothelial

Introduction

Cells, tissues, and organisms are frequently exposed to decreases in environmental oxygen concentration. The ability to tolerate acute hypoxia and, in some cases, to adapt to chronic hypoxia is critical to their survival; however, it varies greatly among different cell types. For example, many invertebrates and lower vertebrates have developed a remarkable tolerance to hypoxia (1–4); in contrast, most mammalian cells and organisms have limited strategies for coping with oxygen depletion and are, consequently, relatively hypoxia sensitive. Yet, even among mammalian cells, there is a marked difference in sensitivity to hypoxia.

Among the numerous mammalian cell types in which tolerance and adaptation to hypoxia are likely crucial to survival are vascular endothelial cells, since they are directly exposed to decreases in blood oxygen tension. In particular, pulmonary artery endothelial cells (PAEC)[1] are continually exposed to the low oxygen content of mixed venous blood (Po_2 35–45 mmHg) and, during pathological states, may be exposed to even lower levels. Under similar conditions in vitro, we and others have demonstrated alterations in many cellular responses in PAEC, but have also noted that these cells retain their viability and cellular integrity (5–11). In contrast, under similar experimental conditions, other mammalian cell types, such as cardiac myocytes, hepatocytes, renal tubular epithelial cells (RTEC), and neurons are extremely hypoxia-sensitive and are rapidly and irreversibly damaged (12–15).

Although little is known about the mechanisms by which endothelial cells, particularly PAEC, maintain cellular and functional integrity under these physiological and pathological conditions, several recent studies have linked their hypoxia tolerance, in part, to the ability to preserve plasma membrane integrity (16) and to underlying modes of energy metabolism (17–19). The latter studies have shown that endothelial cells are predominantly governed by glycolytic metabolism and possess a large capacity to increase this metabolism. For example, endothelial cells exposed to hypoxia increase expression of one of the glucose transporters (19) and enhance glycolytic activity sufficient to maintain relatively normal levels of ATP (17, 19). The former study also suggested that, among cellular membranes, mitochondrial membranes, a major site of nucleotide metabolism, might be important in the endothelial cell response to acute and chronic hypoxia.

Taken together, these studies suggest that the metabolism of purine nucleotides, or perhaps the lack thereof, might play a role in the endothelial cell tolerance to hypoxia. In the current study, we compared the turnover of adenine and guanine nucleotides in hypoxia-tolerant PAEC to the same parameters in hypoxia-sensitive RTEC during exposure to acute hypoxia. Then, because of the disparity between the oxygen tension to which PAEC are exposed in vivo and the oxygen tension in which they are normally cultured, we compared the same parameters in these cells cultured under standard tissue culture conditions (21% oxygen) with cells grown long-term in an oxygen concentration (3% oxygen) similar to that to which they are exposed in vivo. Finally, we examined these parameters in PAEC grown

1. *Abbreviations used in this paper:* PAEC, pulmonary artery endothelial cells; RTEC, renal tubular epithelial cells.

long-term in 3% oxygen and then exposed acutely to 0% oxygen. We found that, unlike hypoxia-sensitive RTEC, hypoxia-tolerant PAEC preserve cellular adenine and guanine nucleotides under various hypoxic conditions. These findings suggest that preservation of these nucleotides may be an important factor in the tolerance of PAEC to acute and chronic hypoxia.

Methods

Materials. MEM and trypsin-EDTA were from GIBCO (Grand Island, NY). Tissue culture plastic was from Falcon Plastics (Los Angeles, CA) and Costar Corp. (Cambridge, MA). Bovine calf serum was from Hyclone Laboratories (Logan, UT). The sealed chambers for hypoxic incubations were from Billups-Rothenburg (Del Mar, CA). Tris was from Bethesda Research Laboratories (Gaithersburg, MD). Adenosine, AMP, ADP, ATP, guanosine, GMP, GDP, and GTP were from Sigma Immunochemicals (St. Louis, MO). Thin-layer DEAE and silical gel chromatography plates were from Whatman Inc. (Clifton, NJ). [³H]-adenosine (40 Ci/mmol) and [³H]guanosine (15 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). [⁸,¹⁴C]adenosine-5'-triphosphate (50 μCi/μmol), chromium-51 (800 Ci/g), and [³,⁵,⁸H]guanosine-5'-triphosphate (1 mCi/mmol) were from New England Nuclear (Boston, MA).

Cell culture. Bovine PAEC were obtained as previously described (9-11, 16). Endothelial cells were harvested by lightly scraping the luminal surface of longitudinally opened pulmonary arteries; the cells were suspended in MEM containing 15% heat inactivated bovine calf serum and initially seeded in 25-cm² flasks or 60-mm plastic dishes. From isolation, the cells were incubated in 95% air, 5% CO_2 at 37°C or in a humidified sealed chamber maintained at 37°C gassed with 3% O_2, 5% CO_2, balance N_2. The cells were maintained in the same oxygen environment throughout their existence in culture (2-4 mo). The chambers were regassed every 24 h; the partial pressure of oxygen in the culture medium was measured using a pH/blood gas analyzer and was similar to that previously reported (9-11, 20). Confluent endothelial cells were passed after treatment with 0.25% trypsin-EDTA. Endothelial cell purity was assessed by phase microscopic "cobblestone appearance," the presence of von Willebrand factor, angiotensin-converting enzyme activity, and uptake of fluorescent acetylated low density lipoprotein (9-11). Experiments were performed using endothelial cells of passage 3-12 from several different primary cell lines.

RTEC were obtained from Dr. Wilfred Lieberthal (Renal Section, Boston University School of Medicine, Boston, MA). For these experiments, an immortalized opossum RTEC line, cultured in MEM containing 15% heat inactivated bovine calf serum and passaged enzymatically was used at passage 50-60.

Experimental design. Adenosine and guanosine uptake, profiles, and metabolism were examined in PAEC under three different hypoxic conditions whereas metabolism of these nucleotides in RTEC was examined in only one hypoxic condition (see below). Initially (Response to Acute Hypoxia), normoxic PAEC or RTEC were exposed to 0% oxygen for 4 h or 18 h and metabolism of these purine nucleotides examined. As a control, the same parameters were examined in PAEC and RTEC maintained in 21% oxygen for the same times. Next (Adaptive Response to Chronic Hypoxia), baseline adenosine and guanosine uptake and profiles were examined in PAEC grown long-term (2-4 mo) in 3% oxygen and compared to the same parameters in PAEC grown in standard culture conditions. Finally (Response of Chronically Adapted Cells to Acute Hypoxia), adenosine and guanosine uptake, profile, and metabolism were examined in chronically hypoxic PAEC exposed acutely to 0% oxygen for 4 or 18 h. As controls, the same parameters were examined in PAEC remaining in 3% oxygen for the same times.

Cell viability. Endothelial and epithelial cell monolayers exposed to hypoxic conditions were assessed for injury by phase microscopic appearance, adherent cell counts, trypan blue exclusion, and chromium-51 release (9, 16, 20, 21).

Purine nucleoside uptake and incorporation. Endothelial and epithelial cell monolayers were incubated with [³H]adenosine or [³H]-guanosine (10 × 10⁶ cpm) during the various hypoxic conditions. After incubation and removal of media, monolayers were rinsed twice with PBS; pH 7.4. The percent uptake of either radiolabel was determined by counting the radioactivity remaining in the media plus the rinses and the radioactivity incorporated into the endothelial or epithelial cell monolayers, and comparing both these values to the total amount of radioactivity initially added to the monolayers.

Analysis of purine nucleotide phosphates. Nucleotide phosphates were extracted from monolayers rinsed previously with PBS with 500 μl 0.5 M $HClO_4$. The cells were collected in glass tubes and centrifuged at 2,500 g at 4°C for 10 min. The pH of the resulting supernatant was increased to 7.0 by addition of 0.5 M KOH (~ 500 μl); this solution was vortexed and placed on ice for 60 min. After being on ice for 60 min, the samples were centrifuged at 8,000 g at 18°C for 5 min; 20 μl of the resulting supernatant was applied to thin layer chromatography (TLC) plates with the appropriate monophosphate, diphosphate, and triphosphate standards (50 μg of each in a total vol of 5 μl). After spotting, the TLC plates were developed using ascending chromatography on DEAE-cellulose with 0.04 M HCl. The TLC plates were air-dried, the nucleotides visualized with ultraviolet light, and the areas corresponding to the adenine nucleotides (ATP [$R_f = 0.19$], ADP [$R_f = 0.55$], AMP [$R_f = 0.90$]) or guanine nucleotides (GTP [$R_f = 0.10$], GDP [$R_f = 0.57$], GMP [$R_f = 0.76$]) scraped, suspended in scintillation fluid, and counted. This method produced consistent separation of nucleotides that was similar in all experiments. As a control, in selected experiments, the cellular content of ATP was determined by the luciferin/luciferase method as previously described (22). Briefly, monolayers were extracted with ice-cold 0.4 M perchloric acid; supernatants of acid extracts were neutralized to pH 7.4 with 4 M K_2CO_3 and ATP levels evaluated. In these experiments, ATP levels were normalized to both cell number and protein content. Cellular content of GTP was not determined by a second method.

Isolation of mitochondria. Mitochondrial fractions were isolated by differential centrifugation using modifications of previous methods (23). After the experimental period, endothelial or epithelial cell monolayers were initially rinsed twice with PBS (pH 7.4) and harvested by rubber policeman. The cells were then disrupted in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) and the homogenate centrifuged in 0.25 M sucrose-0.05 M Tris HCl (pH 7.0) at 800 g for 15 min. The resultant supernatant was centrifuged at 12,000 g for 15 min and the ensuing pellet washed twice in 0.25 M sucrose-0.05 M Tris HCl (pH 7.0). The sediment was treated with acetone at −20°C, and then extracted with 0.05 M Tris HCl (pH 7.4) to obtain soluble ATPase (3.6.1.3) and GTPase (3.6.1.3).

Analysis of mitochondrial ATPase and GTPase activity. 200 μl 0.01 M Tris HCl (pH 7.0) was added to 30 μg of the extracted mitochondrial fraction containing soluble ATPase and GTPase at room temperature; the suspension was gently vortexed and centrifuged at 3,000 g for 5 min. The reaction mixture in a final vol of 100 μl consisted of 20 μl supernatant, 2 μM cold ATP or GTP, 1 μM $MgCl_2$, 50 μM Tris (pH 7.4), and radiolabeled adenosine-5'-triphosphate or guanosine-5'-triphosphate (2 × 10⁵ cpm). This mixture was incubated at 37°C for 60 min, then boiled for 60 s, and centrifuged at 8,000 g for 10 min. 20 μl of the resultant supernatant was applied to TLC plates and developed with the appropriate nucleotide phosphate standards.

Data analysis. Data were expressed as the mean±SEM of the median of five or more experiments for each condition. Each median was derived from five to seven separate monolayers. The nonparametric U-test or one-way analysis of variance followed by Student-Newman-Keul's multiple comparison test was used to compare data. Results were considered significant when $P < 0.05$.

Results

Response to acute hypoxia. PAEC exposed to 0% oxygen for periods up to 18 h demonstrated no evidence of cellular damage.

Table I. Adenosine and Guanosine Uptake and Incorporation into Purine Nucleotide Phosphates of Endothelial and Epithelial Cells during Various Hypoxic Conditions

Cell type	Radioactivity		
	Media	Cells	Purine nucleotide phosphates
	%		
Adenosine			
PAEC (21%)	10	90	5
PAEC (21–0%/4 h)	10	90	6
PAEC (21–0%/18 h)	10	90	11*
RTEC (21%)	20	80	33
RTEC (21–0%/4 h)	20	80	32
RTEC (21–0%/18 h)	40*	60*	28
PAEC (3%)	34‡	66‡	10‡
PAEC (3–0%/4 h)	32‡	68‡	9
PAEC (3–0%/18 h)	50*‡	50*‡	5*‡
Guanosine			
PAEC (21%)	20	80	3
PAEC (21–0%/4 h)	20	80	4
PAEC (21–0%/18 h)	20	80	6*
RTEC (21%)	40	60	20
RTEC (21–0%/4 h)	40	60	20
RTEC (21–0%/18 h)	40	60	19
PAEC (3%)	25	75	10‡
PAEC (3–0%/4 h)	25	75	10‡
PAEC (3–0%/18 h)	35‡	65‡	8

Data are median of five to seven separate experiments and represent percentings of [^{3}H]adenosine and [H]guanosine initially added to cultures that were found in media, cells, or purine nucleotide phosphates after various hypoxic exposures (see Methods for details). *$P < 0.05$ compared to baseline condition in group. ‡$P < 0.05$ chronically hypoxic (3%) cells compared with normoxic (21%) counterpart.

There was no alteration in phase–microscopic appearance, adherent cell counts, trypan-blue exclusion, or chromium-51 release (data not shown). In contrast, RTEC demonstrated marked sensitivity to hypoxia. Similar to primary mouse RTEC (11, 21), immortalized-opposum RTEC demonstrated significant cellular injury after 18 h of hypoxic exposure (pyknotic cells with trypan exclusion 60%, adherent cell counts 41%, chromium release 165% of normoxic RTEC).

Exposure to acute hypoxia for 4 h caused no significant changes in the uptake and incorporation of radiolabeled adenosine or guanosine in either endothelial cells or renal tubular epithelial cells (Table I). In contrast, exposure to longer periods of hypoxia (18 h) modified the uptake and incorporation of radiolabeled adenosine or guanosine in both endothelial cells and renal tubular epithelial cells; however, these alterations were different depending on the cell type (Table I). In PAEC, despite no change in the uptake of either nucleoside after exposure to acute hypoxia for 18 h, incorporation into purine nucleotide phosphates increased twofold in both cases. Thus, the amount of exogenous adenosine or guanosine incorporated into purine nucleotide phosphates was significantly higher in hypoxic PAEC than in their normoxic counterparts. In RTEC, up-

Table II. Purine Nucleotide Phosphate Profiles in Endothelial and Epithelial Cells during Various Hypoxic Conditions

Cell type	ATP	ADP	AMP	GTP	GDP	GMP
PAEC (21%)	5	35	60	12	71	17
PAEC (21–0%/4 h)	5	38	57	12	70	18
PAEC (21–0%/18 h)	4	38	58	17*	60*	23
RTEC (21%)	60	23	17	40	15	45
RTEC (21–0%/4 h)	60	22	18	40	15	45
RTEC (21–0%/18 h)	13*	73*	14	20*	10	70*
PAEC (3%)	28‡	45‡	27‡	9‡	85‡	6‡
PAEC (3–0%/4 h)	27‡	46‡	27‡	10	86‡	4‡
PAEC (3–0%/18 h)	22‡	36*	42*‡	18*	68*	14*‡

Data are median for five to seven separate experiments and represent percentages of [^{3}H]adenosine and [^{3}H]guanosine initially added to cultures that were found in purine nucleotide phosphates after various hypoxic exposures (see Methods for details). *$P < 0.05$ compared to baseline condition in group. ‡$P < 0.05$ chronically hypoxic (3%) cells compared with normoxic (21%) counterpart.

take of adenosine was significantly decreased by exposure to acute hypoxia for 18 h; uptake of guanosine did not change. In contrast to the increase in hypoxic PAEC, incorporation of either nucleoside into purine nucleotide phosphates was not changed.

Purine nucleotide phosphate profiles and measured ATP levels were markedly different in PAEC and RTEC, not only in normoxia, but also following exposure to acute hypoxia (Tables II and III). In normoxic PAEC, much less radioactivity was found within ATP and GTP and measured ATP levels were lower than in normoxic RTEC. Exposure of PAEC to 0% oxygen for 4 or 18 h did not change the ratio of radiolabeled ATP/ADP/AMP nor the absolute level of ATP; at 18 h, the ratio of radiolabeled GTP increased by a small, but significant, amount. In hypoxic RTEC, the ratio of radiolabel in ATP and GTP did not change after 4 h of hypoxia. After an 18-h exposure, how-

Table III. ATP Levels in Endothelial and Epithelial Cells during Various Hypoxic Conditions

Cell type	ATP
	nmol/mg protein
PAEC (21%)	6.2±0.9
PAEC (21–0%/4 h)	6.4±0.6
PAEC (21–0%/18 h)	5.8±1.1
RTEC (21%)	45.2±6.3
RTEC (21–0%/4 h)	42.1±4.6
RTEC (21–0%/18 h)	6.9±2.0*
PAEC (3%)	11.6±1.8‡
PAEC (3–0%/4 h)	10.8±1.3‡
PAEC (3–0%/18 h)	10.4±1.0‡

Data are mean ± SEM of five to seven determinations by the luciferin/luciferase method (see Methods for details). *$P < 0.05$ compared to baseline condition in group. ‡$P < 0.05$ chronically hypoxic (3%) cells compared with normoxic (21%) counterpart.

Table IV. Activity of mitochondrial ATPase and GTPase
in Endothelial and Epithelial Cells during Various
Hypoxic Conditions

Cell type	ATPase activity	GTPase activity
PAEC (21%)	0.40	0.65
PAEC (21–0%/4 h)	0.18*	0.33*
PAEC (21–0%/18 h)	0.17*	0.18*
RTEC (21)	0.32	0.44
RTEC (21–0%/4 h)	0.15*	0.21*
RTEC (21–0%/18 h)	0.03*	0.04*
PAEC (3%)	0.07‡	0.10‡
PAEC (3–0%/4 h)	0.08‡	0.09‡
PAEC (3–0%/18 h)	0.07‡	0.09‡

Data are median of five to seven separate experiments and represent activity of mitochondrial ATPase and GTPase (μM inorganic ortho-phosphate/mg protein per 60 min) after various hypoxic exposures (see Methods for details). *$P < 0.05$ compared to baseline condition in group. ‡$P < 0.05$ chronically hypoxic (3%) cells compared with normoxic (21%) counterpart.

ever, the ratio of radiolabeled ATP and absolute-ATP levels were ~ 20% of that during exposure to either normoxia or 4 h of hypoxia; the ratio of radiolabeled GTP decreased to 50% of those values. In each case, there was an increase in the ratio of the corresponding diphosphated or monophosphated nucleotides. Thus, in hypoxic PAEC, ATP and GTP profiles were maintained whereas these nucleotides were extensively depleted by exposure to hypoxia in RTEC.

Exposure to 0% oxygen for 4 h significantly decreased the activity of both mitochondrial ATPase and GTPase in PAEC (Table IV). There was no further decrease in the activity of ATPase after an 18-h exposure, whereas the activity of GTPase continued to decrease with the longer exposure. In RTEC, mitochondrial ATPase and GTPase activities were even more sensitive to acute hypoxia, especially at the 18-h time point (Table IV). The activity of both enzymes decreased at 4 h and had diminished precipitously at the 18-h time point.

Adaptive response to chronic hypoxia. Similar to previous reports, PAEC cultured long-term in 3% oxygen proliferated, retained the characteristics associated with cultured endothelial cells, and demonstrated no evidence of cellular injury (9–11, 16).

Compared to their normoxic counterparts, chronically hypoxic PAEC demonstrated a significant decrease in the uptake of adenosine and no change in the uptake of guanosine (Table I). However, with either nucleoside, the amount of radioactivity found in purine nucleotide phosphates after long-term culture in 3% oxygen increased significantly.

Purine nucleotide phosphate profiles in these chronically hypoxic cells had also changed significantly from that of their normoxic counterparts (Tables II and III). Of note, there was a fivefold increase in the ratio of radiolabeled ATP, a twofold increase in absolute ATP levels, a small, but significant, decrease in the ratio of radiolabeled GTP, as well as an increase in the ratio of diphosphated nucleotides, and a marked decrease in the ratio of monophosphated nucleotides in these cells.

In chronically hypoxic PAEC, the activity of mitochondrial ATPase and GTPase was significantly less at baseline than in normoxic PAEC (Table IV).

Response of chronically adapted cells to acute hypoxia. PAEC cultured long-term in 3% oxygen, then exposed acutely to 0% oxygen for periods up to 18 h demonstrated no alteration in phase microscopic appearance, adherent cell counts, trypan-blue exclusion, or chromium-51 release (data not shown).

Under these conditions, the uptake and incorporation of either nucleoside was not changed by a 4 h exposure (Table I). After 18 h, uptake of either nucleoside had decreased. In addition, with adenosine as the substrate, the amount of radioactivity found in purine nucleotide phosphates decreased; with guanosine as the substrate, there was no significant change in radiolabeled purine nucleotide phosphates.

In chronically hypoxic PAEC exposed acutely to 0% oxygen for 4 h, there was no change in the profile of adenosine or guanosine phosphates (Tables II and III). After an 18-h exposure, no change in the ratio of ATP nor its absolute level was found, but there was an increase in the ratio of radioactivity appearing in GTP. In addition, at this time point there were significant decreases in the ratio of diphosphates and increases in the ratio of monophosphates of each nucleotide.

The activity of mitochondrial ATPase and GTPase activity in chronically hypoxic PAEC was not changed by acute exposure to 0% oxygen for either 4 or 18 h (Table IV).

Discussion

In hypoxia-sensitive tissues and cells, glucose and oxygen consumption are inversely related (Pasteur effect); under conditions in which oxygen is limited, there is a dramatic rise in the demand for glucose to maintain ATP turnover at a level similar to that during normoxia (1). Theoretically, because of this energy insufficiency and increasing membrane permeability, electric and ion potentials cannot be maintained and progressive uncoupling of metabolic and membrane functions ensues, eventually leading to progressive damage and cell death. To obviate these problems and to extend hypoxia tolerance, there are several potentially useful protective measures that hypoxia-tolerant organisms, tissues, and cells could use: (*a*) large stores of fermentable fuel; (*b*) high tolerance of metabolic acidosis and/or compensatory ion changes that retard the development of acidosis; (*c*) metabolic rate depression; and (*d*) improved handling of intracellular calcium. Among these potential strategies, several recent studies have indicated that the most effective are metabolic rate suppression and reduced membrane permeability (24–28). By virtue of these strategies (termed metabolic and channel arrest), hypoxia-tolerant organisms, tissues, or cells would not have to compensate for the energy deficit arising from a lack of oxygen. Thus, despite lower energy turnover, they could retain coupled metabolic and membrane functions by maintaining membranes of low permeability (27, 28). In addition, by decreasing metabolic demands they would decrease the need for ATP, preserve energy substrate, and limit buildup of toxic or acidic metabolic end-products during periods of oxygen deprivation (24–26, 29).

Moreover, it has been suggested that most, if not all, hypoxia-tolerant tissues suppress ATP turnover rates drastically during oxygen limitation so that there is minimal change in the concentration of phosphate metabolites or in the phosphorylation potential (29). Under these conditions, declining demands

for ATP would be so closely matched by ATP synthesis rates that the two processes would stabilize cell nucleotide concentrations. In fact, coupling of energy demand to supply is a highly adaptable characteristic and has been demonstrated in several species (30–32). Whether the capacity for, and the degree of, this adaptation (metabolic arrest) is similar in all tissues is, as yet, unclear. Nevertheless, it is considered by some investigators to be the critical mechanism allowing survival without oxygen (29, 30, 32). Whether similar mechanisms for preservation of guanine nucleotide phosphates exist and/or whether maintenance of these nucleotide concentrations is important in hypoxia-tolerant tissues or cells has not been determined.

Among the numerous cell types in which tolerance and adaptation to hypoxia are likely crucial to survival are vascular endothelial cells, since they are frequently and directly exposed to decreases in blood oxygen tension. Although one would expect other cell types that have higher energy demands and experience lower oxygen tensions to be as, if not more, hypoxia tolerant than endothelial cells, this has not been established. In fact, numerous investigators have demonstrated that these cells, e.g., myocardial cells, hepatocytes, neurons, and renal tubular epithelial cells, are extremely hypoxia sensitive, much more so than endothelial cells (9, 12–15, 20, 21); however, the reason(s) and mechanism(s) for this dichotomy are not yet understood. Among endothelial cells, PAEC survive and function in decreased ambient oxygen (Po_2 of 35–45 mmHg) and, during pathological states may be exposed to even lower levels. Little is known about the mechanisms by which these cells maintain cellular and functional integrity under these physiological and pathological conditions. To elucidate the effects of such levels of ambient oxygen, we and others have used cultured PAEC to examine physiological and pathological levels of oxygen both acutely and chronically (5–11, 20, 21). Studies of these cells exposed to short-term (1–5 d) and long-term (1–4 mo) hypoxia have demonstrated alterations in many cellular responses, but have also demonstrated retention of cellular integrity and viability (5–11, 20, 21). Although little is known about how PAEC maintain their cellular functions in these conditions, recent studies have demonstrated that during short-term hypoxia ATP levels are maintained (1, 19, 33). Furthermore, other studies have suggested that endothelial hypoxia tolerance is related to underlying modes of energy metabolism (17–19). These latter studies have demonstrated that endothelial cells operate under mainly glycolytic metabolism and possess a large capacity to increase this metabolism. For instance, in endothelial cells exposed to hypoxia, glycolytic activity is enhanced to a level sufficient to maintain relatively normal levels of ATP (17, 19). Taken together, these studies suggest that the preservation of ATP may be important in the development of hypoxia tolerance in cultured endothelial cells. In contrast, in hypoxia-sensitive cell types, there is a rapid depletion of ATP after exposure to hypoxic or ischemic conditions (1, 15, 29, 34). Again, the potential importance of guanine nucleotides in the development of hypoxia tolerance has not been determined; however, a recent study demonstrated preserved GTP levels in cultured hepatocytes from the anoxia-tolerant western painted turtle during a 12 h exposure to 0% oxygen (24).

The current study, the first to compare the metabolism and profiles of both adenine and guanine nucleotides during graded hypoxic exposures between hypoxia-tolerant and hypoxia-sensitive mammalian cells, expand the theory that hypoxia tolerance

may be linked to preservation of nucleotide concentrations. Under several different hypoxic conditions, hypoxia-tolerant PAEC maintained or, in some cases, actually increased the absolute levels of ATP and the percentage of these nucleotides found in the high energy phosphates, ATP, and GTP. Moreover, the percentage of nucleotides found in ATP and its absolute level were even higher, both at baseline and during acute exposure to 0% oxygen, in endothelial cells grown long-term in 3% oxygen than in their normoxic counterparts. These findings suggest that these mammalian cells not only have the capacity to maintain nucleotide profiles during acute hypoxia, but also are able to sustain this capability and, seemingly, augment it despite exposure to chronic hypoxia. In contrast, in hypoxia-sensitive RTEC, the absolute level of ATP and same high energy phosphates (ATP and GTP) were rapidly depleted. This differential response in nucleotide profiles was associated with minor changes in the uptake of the precusor nucleoside in both cell types, but with increased incorporation into the appropriate purine nucleotide phosphates only in hypoxic PAEC. Furthermore, although decreases in PAEC ATPase and GTPase activity under these conditions would suggest, at first appearance, that a reduction in degradation of these high energy phosphates is another possible mechanism for this preservation, the situation is likely much more complex. First, even greater decreases in RTEC ATPase and GTPase were noted during hypoxia. Second, in vivo, ATPase (usually designated ATP synthase) synthesizes ATP from ADP; however, when mitochondria are disrupted and submitochondrial particles isolated in vitro, such as in the current study, the enzyme functions as an ATPase and hydrolyzes ATP. Furthermore, there is increasing evidence that ATP synthase in vivo can also hydrolyze ATP rather than synthesize it. Whether this enzyme functions in its ATP-synthesizing or its ATP-hydrolyzing direction at any instant depends on the concentration of ATP, ADP, and inorganic orthophosphate in the mitochondrial matrix space and the value of the proton-motive force across the inner mitochondrial membrane (35, 36). Unfortunately, the effect of hypoxia on each of these components in endothelial cells or other cell types is not yet known. Because, in most cell types, exposure to hypoxia causes a rapid drop in ATP concentrations, it is presumed that hydrolysis is the predominant function of the enzyme under these conditions (35, 36). Yet, it is certainly possible that this drop in ATP concentration is due to a rapid increase in utilization of ATP with concomitant inhibition of the ATP-synthesizing function of ATP synthase. Nonetheless, we have used current convention and have assumed that the enzyme complex isolated from submitochondrial particles functions as ATPase and hydrolyzes ATP. Third, ATP and GTP levels are a complex relationship between utilization, synthesis, and degradation (25). Thus, in RTEC, use of existent ATP and/or GTP and inability to synthesize these high energy phosphate molecules, either by an increase in glycolysis or by other pathways, could overwhelm the attempt by these cells to decrease degradation. In contrast, in PAEC, the reduction in degradative activity alone or, more likely, combined with metabolic arrest and/or new synthesis of high energy phosphates is sufficient to preserve these molecules. This preservation of high energy phosphates in hypoxic PAEC suggests very tight regulation of ATP and GTP turnover in these cells, possibly by a single regulatory signal (29). What that signal might be, or whether that signal even exists, is currently unknown. Further studies will be necessary to clarify

these possibilities. However, a recent finding may be of interest in this regard.

We and others have examined the proteins induced after exposure to varying lengths and intensities of hypoxia in a variety of mammalian cells. Most cell types studied respond to hypoxia by inducing heat shock and/or glucose-regulated proteins (37–41). These proteins are not specific to hypoxia and are upregulated or induced in response to other cellular stresses, such as increased temperature and glucose deprivation. Our laboratory has shown that endothelial cells do not upregulate heatshock and/or glucose-related proteins in response to acute or chronic hypoxia, but instead upregulate a unique set of five proteins, termed hypoxia-associated proteins (11). Further studies have identified the 36 kD hypoxic-associated protein as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (42). Of potential interest to the current study, this enzyme catalyzes an oxidation-reduction reaction in which NAD+ is reduced to NADH, glyceraldehyde 3-phosphate is oxidized, and a high energy phosphate compound is generated (43). Moreover, although not the primary regulatory site for glycolysis, GAPDH may play a secondary regulatory role in conjunction with phosphoglycerate kinase, the enzyme catalyzing the ATP generating reaction immediately after GAPDH (44, 45). Of further interest, upregulation of GAPDH is specific to hypoxic stress and has not been demonstrated in other mammalian cell types, including hypoxia-sensitive RTEC (21). Thus, it is possible that GAPDH is upregulated in hypoxic PAEC as a mechanism to increase the synthesis of ATP and that a similar mechanism exists in these cells for synthesis of GTP.

In summary, the current study is the first to demonstrate preservation of adenine and guanine nucleotides in hypoxia-tolerant PAEC during exposure to acute and chronic hypoxia. In contrast, in hypoxia-sensitive RTEC, there was rapid and dramatic depletion of the same nucleotides associated with irreversible cell damage. In addition, this is the initial study to suggest that metabolic arrest, an increasingly reported mechanism for developing hypoxia tolerance in invertebrates and lower vertebrates, may also occur in some mammalian cells. Although further studies will be necessary to confirm these suggestions and to determine whether this preservation of high energy phosphates is sufficient, in and of itself, to confer hypoxia tolerance, it is not improbable that other measures, such as the lack of plasma membrane hydrolysis (16) and the upregulation of a specific set of stress proteins (11, 21, 42), may also contribute to the ability of PAEC to tolerate acute and chronic decreases in ambient oxygen concentration.

Acknowledgments

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