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Receptor-Stimulated Phospholipase C Activity in Human Umbilical Artery Cultured Endothelial Cells Grown in a Low Oxygen Environment

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ABSTRACT. Endothelial cells of the human umbilical blood vessels are widely cultured in an oxygen tension (21%) far above that in which they exist *in vivo* (3%). This study investigates the effect of the long term culture (ca. 1 month) of human umbilical artery endothelial cells in a reduced oxygen environment (3%: HUAEC3) in comparison to cells grown in a 'normoxic' environment (21%: HUAEC21). Despite reports of altered metabolic pathways and reduced membrane integrity in other cell types, the characteristics of HUAEC3 were found to be similar to those of HUAEC21 with respect to morphology, immunocytochemical profile and *in vitro* growth rates. Cellular glutathione was maintained in these cells although ATP levels in HUAEC3 were found to be significantly lower than those observed in HUAEC21. The phosphoinositide responses of the HUAEC3 to a variety of agonists were also found to be of similar magnitude to those observed in HUAEC21. In addition, the pharmacological characteristics of the phospholipase C-linked histamine H_1 and P_{2y2} (P_{2U}) receptors were not changed by culture of cells in a low oxygen environment. BIOCHEM PHARMACOL **54**;12:1351–1359, 1997.

KEY WORDS. phosphoinositide hydrolysis; oxygen tension; vascular endothelial cells; physical properties

Endothelial cells taken from the blood vessels of the human umbilical cord are used in many areas of research because of the relative ease with which they can be isolated. However, the vessels of the umbilical cord are unusual in that they must remain patent *in utero* at a very low oxygen tension [1] but retain the ability to close quickly and completely after delivery when they are exposed to higher levels of oxygen [2]. These endothelial cells therefore survive in utero at an oxygen tension far below the 21% atmospheric oxygen in which they are routinely grown in culture [3, 4].

On the fetal side of the placenta the oxygen tension (pO_2) in the human umbilical artery is approximately 16 mmHg [1] which would be considered to be severely hypoxic in other vascular beds. Studies involving endothelial cells from various vascular beds have revealed that the glucose transporter GLUT-1 [5], platelet derived growth factor B chain [6], endothelin [7], vascular endothelial growth factor [8], and constitutive nitric oxide synthase [9] can be induced by hypoxia. There is also an increase in the expression of genes encoding a group of five proteins that are distinct from heat shock proteins known as hypoxia associated proteins (HAP [10])§, one of which has been identified as glyceraldehyde-3-phosphate dehydrogenase [11]. However, it is unclear at the present time whether or not intracellular signal transduction mechanisms are altered at low oxygen tensions. Exposure of endothelial cells to reduced environmental oxygen levels leads to alterations in many membrane-dependent functions which may be a consequence of alterations in membrane phospholipids [12, 13]. For example, it has been shown that changes in cyclooxygenase metabolism in aortic and pulmonary arterial endothelial cells exposed to acute and chronic hypoxia can, in part, be explained by alterations in membrane phospholipids and/or membrane phospholipase activity [14, 15].

The human umbilical artery is devoid of innervation [16, 17] and therefore control of the vessel's tone is predominantly mediated via the release of vasoactive substances produced locally or conveyed through the blood stream. The endothelial cells of the human umbilical vessels play an important role in the control of vascular smooth muscle tone via the production of prostacyclin [4, 18], endothelium-derived relaxing factor (NO [19]) and endothelin [7]. We have previously demonstrated that several agonists,

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§ *Abbreviations:* BPAEC, bovine pulmonary artery endothelial cells; CHO cells, chinese hamster ovary cells; FCS, fetal calf serum; HAP, hypoxia associated proteins; HUAEC, human umbilical artery endothelial cells; HUAEC21, human umbilical endothelial cells grown at 21% oxygen; HUAEC3, human umbilical artery endothelial cells grown at 3% oxygen.

including histamine, ATP or thrombin can induce an increase in phosphoinositide (PI) hydrolysis in human umbilical artery endothelial cells (HUAEC [3]), which can produce subsequent effects on intracellular calcium ion mobilisation [20, 18] and the synthesis and release of the arachidonic acid metabolite prostacyclin [21, 22]. Therefore one of the aims of this study was to compare the phosphoinositide responses induced by these agonists in HUAEC grown at different oxygen tensions.

The vast majority of studies reporting cellular changes have investigated the effects on cells of only short periods of hypoxic exposure (6–48 hr [5, 7, 8, 11, 23]). In the light of the changes observed in these reports we have evaluated the integrity of HUAEC maintained for several passages in a low oxygen environment. Here we describe the isolation and long term maintenance of human umbilical arterial endothelial cells in a 3% oxygen atmosphere. These cells have been characterised with respect to their morphology, immunocytochemical profiles, growth rates, cellular ATP and glutathione levels, and phosphoinositide responses. These properties have been compared to those obtained from HUAEC grown in a 21% atmosphere from isolation.

MATERIALS AND METHODS

Chemicals

Collagenase (Type II) and trypsin were obtained from Worthington Biochemicals. Myo[^{3}H]inositol was from NEN Life Science Products. Human serum and endothelial cell growth supplement were obtained from Advanced Protein Products. All other chemicals and antibodies were obtained from the Sigma Chemical Co.

Cell Culture

Human umbilical artery endothelial cells were isolated essentially as described previously [3]. Briefly, lengths of umbilical cords obtained after delivery were cannulated at both ends using cut down vessel dilators. The artery was then washed with isotonic saline solution to remove red blood cells and filled with 0.5 mg/mL collagenase (type II) in medium 199 at 37°. The umbilical cord was then incubated in a saline bath maintained at 37° for a further 5 min. Endothelial cells were then removed from the artery by flushing through with medium 199 which was then placed in an equal volume of foetal calf serum. The cell suspension was centrifuged for 10 min at 100 × *g* and the resulting pellet was re-suspended in 5 mL of medium 199 containing 10% human serum, 10% fetal calf serum, 2 mM glutamine, 20 μg/mL endothelial cell growth supplement, 90 μg/mL heparin, 100 U/mL penicillin G, 100 μg/mL streptomycin and 250 ng/mL amphotericin B. Cells were then grown in either a 21% O_2 (HUAEC21) or a 3% O_2 (HUAEC3) environment maintained at a humidified 37° with 5% CO_2. All other manipulations of HUAEC3 were performed in a custom built low oxygen microbiological safety cabinet which provides an atmosphere of 3% O_2 by displacement with nitrogen gas. Media for HUAEC3 was pre-gassed in a 3% O_2 environment for several hours before use. Confluent flasks were passaged using 0.05% trypsin in versene (Glasgow formula) into the above media without the antibiotics. A split ratio of 1:3 was used for routine passage and all flasks and multi-well plates were coated with 1% gelatin before use. Characterization was performed on cells at passages 3 and 4 only.

Immunocytochemical Analysis

Indirect immunocytochemistry was used to confirm the identity of the endothelial cells. Briefly cells were grown on 22 mm × 22 mm glass coverslips in medium 199 containing 10% human serum, 10% foetal calf serum, 2 mM glutamine, 25 mg/mL endothelial cell growth supplement and 90 μg/mL heparin until confluency. Cells were then washed (3 × 5 min in phosphate buffered saline, (PBS) and fixed for 10 min at −20° in ice cold methanol. Coverslips were then washed again (3 × 5 min in PBS) and incubated for 30 min with 10% FCS in PBS at room temperature. Polyclonal antibody to von Willebrand factor made up in 10% FCS in PBS or a control solution of 10% horse serum in PBS was then incubated with the cells overnight at 4°. Cells were again washed (3 × 5 min in PBS) and exposed to the second layer of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:20 in 10% horse serum in PBS). Cells were viewed under a Zeiss epifluorescet photomicroscope III using the 487710 filter set.

Growth Characteristics

Replicate numbers of HUAEC3 or HUAEC21 (1.4×10^4 cells/cm^2) were plated out into 6-well plates and cell numbers were estimated in triplicate on days 0, 2, 4, 6 and 8 using a fluorimetric DNA assay. During this time period cells were fed daily by a complete medium change. This method was a modification of that used by Karsten and Wollenberger [24] and is based on the linear relationship between the fluorescence of ethidium bromide when bound to DNA and the concentration of DNA present in the sample within the range of 0–20 μg/mL. A standard curve was constructed for each assay performed using calf thymus DNA at concentrations ranging from 0–20 μg/mL in phosphate buffered saline (PBS) containing 20 μg/mL pronase. Samples for assay were prepared in the following way. 1.0 mL of pronase (36 μg/mL in PBS) was added to each well and then incubated at 37° for 30 min to detach the cells from the plastic. Cell suspensions from each well were then transferred into individual tubes and the remainder of the assay was performed on ice. Wells were rinsed with 800 μL of PBS which was transferred to the corresponding tube and the cells were homogenized for 30 sec/well using a Polytron homogenizer with a 7 mm probe. Duplicate aliquots of 800 μL of standard DNA solutions or the prepared samples were placed in polystyrene tubes for assay. To each tube, 100 μL ribonuclease A (125 μg/mL in

PBS) and 100 μL pronase (115 μg/mL in PBS) were added. The tubes were then vortex mixed and incubated in a sliding water bath at 37° for 1 hr. Following incubation, 500 μL of ethidium bromide (15 μg/mL in PBS) was added to each tube and again vortex mixed. The fluorescence of all the standards and samples was measured on a Perkin Elmer SP2 fluorimeter (540 nm excitation:590 nm emission). DNA concentrations of the samples were calculated by linear regression analysis of the DNA standards.

Determination of Cellular ATP Content

HUAEC3 or HUAEC21 were plated out at a cell density of 1.4×10^4 cells/cm^2 into 24-well plates and grown until day 4. Media was removed from the cells which were then washed gently with 1 mL PBS. Cellular ATP was extracted from the cells by addition of 1 ml perchloric acid (3% v/v) to each well and placing the plates on a rolling platform for 30 min. Acid extracts were then transferred to Eppendorf tubes and neutralised with approximately 120 μL of alkali (7.5M KOH/50 mM K_2HPO_4) and spun for approximately 15 sec in a microfuge. One hundred μL of supernatant was removed and mixed with 200 μL of 0.05M Tris pH 7.7/4 mM $MgSO_4$. Samples were then stored at this stage in liquid nitrogen for up to 3 weeks before determination of ATP content. Cellular ATP was measured by bioluminescence in an LKB Wallac 1250 luminometer as instructed in the LKB Wallac ATP monitoring kit.

Measurement of Cellular Glutathione Levels

HUAEC3 or HUAEC21 were plated out at a cell density of 1.4×10^4 cells/cm^2 into 6-well plates and grown until day 4. At this point cells were washed three times with PBS and then 0.5 mL sulphosalicylic acid (SSA; 15% w/v) was carefully added to each well and gently mixed. The SSA extracts were then transferred to polypropylene tubes and stored at −20° until assay according to the method of Saville [25]. Briefly, 200 μL samples from each extract were taken, mixed with 50 μL solution A (1 volume sodium nitrite 1.2 mg/mL in H_2O:4 volumes 1 M sulphuric acid) and left to stand at room temperature for 5 min. Twenty μL of ammonium sulphamate (10 mg/ml in H_2O) was added to each sample, mixed and allowed to stand for 2 min. This was followed by the addition of 100 μL of solution B (2 volumes sulphanilamide 69 mg/mL in 0.4 M HCl:1 volume mercuric chloride 10 mg/mL in 0.4M HCl) and 125 μL of naphthylethylenediamine (2 mg/mL in 0.4 M HCl) and the colour allowed to develop for 10 min. Volumes of 200 μL from each sample were transferred into wells of a 96-well plate which was then read on a plate reader using a 570 nm filter to measure and a 655-nm filter as reference.

Accumulation of [^{3}H]Inositol Phosphates

In order to measure the production of total [^{3}H]inositol phosphates, HUAEC3 or HUAEC21 were grown to confluency in 24-well plates. Cells were then incubated for 24 hr at 37°C with myo[^{3}H]inositol (1 μCi/well) in 0.3 mL medium 199 supplemented with 1% FCS, 1% human serum and 2 mM glutamine. The media was then removed and cells were washed twice in 1 mL/well Hank's/HEPES (9.76 g/L:20 mM, pH 7.4). Following this cells were incubated for 20 min at 37° in 290 μL Hank's/HEPES containing 20 mM lithium chloride. Where appropriate, antagonists were added at the start of this incubation. Agonists were added in 10 μL of Hank's/HEPES with LiCl for incubation times of 5 to 60 min. Stimulation was stopped by the removal of agonists and the addition of 1 mL ice cold methanol/0.12 M HCl (1:1). Assays involving HUAEC3 were performed with pre-gassed Hanks/HEPES in the low oxygen microbiological safety cabinet under a 3% oxygen environment. Cells were left overnight at −20° before neutralisation with 25mM Tris:0.5M NaOH:H_2O (11:1.2:34). Total inositol phosphates were finally separated from free myo[^{3}H]inositol by anion exchange chromatography [26]. Tritium was determined by liquid scintillation counting.

Data Analysis

Concentration response curves were fitted to a Hill equation using the non-linear curve-fitting programme INPLOT4 (Graphpad Software, CA). The equation fitted was

$$\text{Response} = E_{max} \times D^n/(D^n + (EC_{50})^n)$$

where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half maximal response and E_{max} is the maximal effect. Apparent dissociation constant (K_D) of the receptor antagonist was determined, assuming competitive antagonism, by one of two methods; 1) from shifts in the agonist concentration-response curves using the relationship

$$K_D = D/(K_2/K_1 - 1)$$

where D is the concentration of antagonist, K_1 is the concentration of agonist producing half maximal response and K_2 is the concentration of agonist producing the same response in the presence of antagonist; 2) using a modification of the null method described by Lazareno and Roberts [27]. Briefly, a concentration-response curve to histamine was generated and a concentration (C; 100 μM) of histamine was chosen which gave a response greater than 50% of the maximum histamine response. The IC_{50} required to reduce the response of 100 μM histamine by 50% was then determined. The agonist concentration-response curve was fitted to a logistic equation as described above and a concentration of histamine identified which yielded a response equivalent to 50% of 100 μM histamine (in the absence of antagonist; C′). The apparent K_D was then determined from the relationship:

$$C/C' = IC_{50}/K_D + 1$$

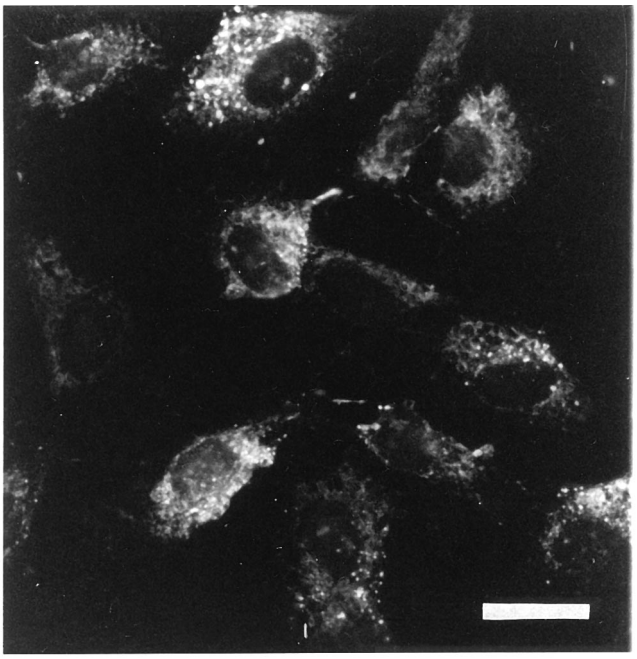

FIG. 1. Human cultured umbilical artery endothelial cells, grown in 3% oxygen, stained for von Willebrand factor by indirect immunocytochemical methods. Cells were incubated overnight at 4° with a polyclonal antibody to von Willebrand factor before exposure to a second fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Bar = 30 μm.

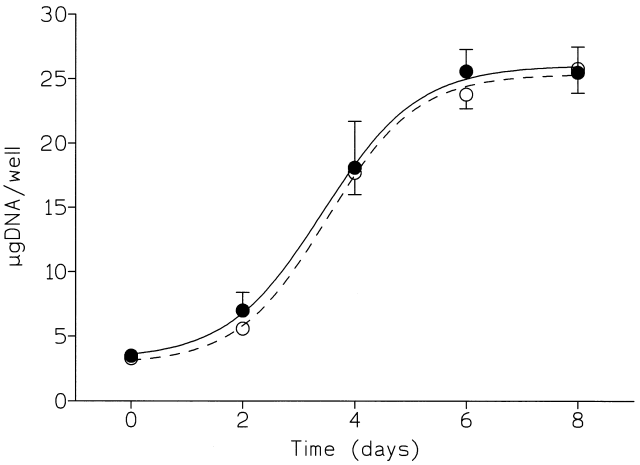

FIG. 2. Growth curves of HUAEC21 (●) and HUAEC3 (○) over eight days. Cells were grown in 6-well plates and growth is expressed as micrograms of DNA per well. Each point represents the mean ± SEM from triplicate determinations in four separate experiments using cells from different donors in each. All cells were at passage 3.

pK_D values were calculated as $-\log K_D$. Statistical analysis was performed by analysis of variance or unpaired *t*-tests. Triplicate or quadruplicate determinations (i.e., separate culture wells) were made in each experiment and, unless otherwise stated, the mean ± SE mean cited in the text refer to the mean data obtained from *n* different experiments using cell lines arising from different donors (i.e., *n* represents *n* different cell lines).

RESULTS

Both HUAEC21 and HUAEC3 exhibited the typical cobblestone morphology of endothelial cells for at least 4 passages. Confirmation of endothelial cell identity was given by positive immunofluorescence staining of HUAEC3 and HUAEC21 using the polyclonal antibody to von Willebrand factor (e.g. Fig. 1). The growth rates of HUAEC3 and HUAEC21 were very similar at passage 3 as assessed by the DNA content of the wells (Fig. 2) reaching the same level of cell density within the culture vessels. HUAEC3 grown in a 3% oxygen environment maintained a similar level of cellular glutathione (2.08 ± 0.32 nmol/10^6 cells) compared to that in HUAEC21 (2.60 ± 0.30 nmol/10^6 cells), there being no statistically significant difference between the two values (Table 1). However cellular ATP levels were found to be significantly lower in HUAEC 3 than in HUAEC21 ($P < 0.05$; Table 1). Glutathione and ATP levels are expressed as nmol/10^6 cells where cell numbers were estimated from the DNA content found in replicate wells on the day of extraction (day 4) assuming a DNA content of 5 pg per cell [28].

Agonist-Induced [^{3}H]Inositol Phosphate Accumulation in HUAEC

Figure 3 shows the effects of various agonists on phosphoinositide hydrolysis in either HUAEC21 (Fig. 3a, b) or HUAEC3 (Fig. 3c, d). Both HUAEC3 and HUAEC21 demonstrated an increase in phosphoinositide turnover in response to 0.1 mM histamine, 1 mM ATP, 1 U/mL thrombin or 20 mM NaF. The responses under the two conditions were not significantly different from each other for each of these agonists. Neither HUAEC3 or HUAEC21 responded to the smooth muscle spasmogens endothelin-1 (1 μM), bradykinin (1 μM) or angiotensin II (10 μM). Very small responses were observed in HUAEC3 cells in response to carbachol (1 mM; $P < 0.01$; 2-way analysis of variance) and 5-hydroxytryptamine (100 μM; $P < 0.05$) which did not reach significance in HUAEC21 cells (Fig. 3).

TABLE 1. Cellular ATP and glutathione content of HUAEC3 and HUAEC21

Cell type	Glutathione nmol/10^6 cells	ATP nmol/10^6 cells
HUAEC3	2.08 ± 0.32 (N = 4)	14.4 ± 1.4* (N = 4)
HUAEC21	2.60 ± 0.30 (N = 4)	21.6 ± 2.0 (N = 4)

Values represent the mean ± SEM in cells from four different donors at each oxygen concentration. For each cell line (obtained from a separate donor) five (ATP) or six (glutathione) replicate measurements were made. All cells were at passage 3. Human umbilical artery endothelial cells were grown in a 3% (HUAEC3) or a 21% (HUAEC21) oxygen environment as described in "Materials and Methods." Cellular glutathione and ATP were extracted four days after plating out cells.

* $P < 0.05$ vs. 21% oxygen.

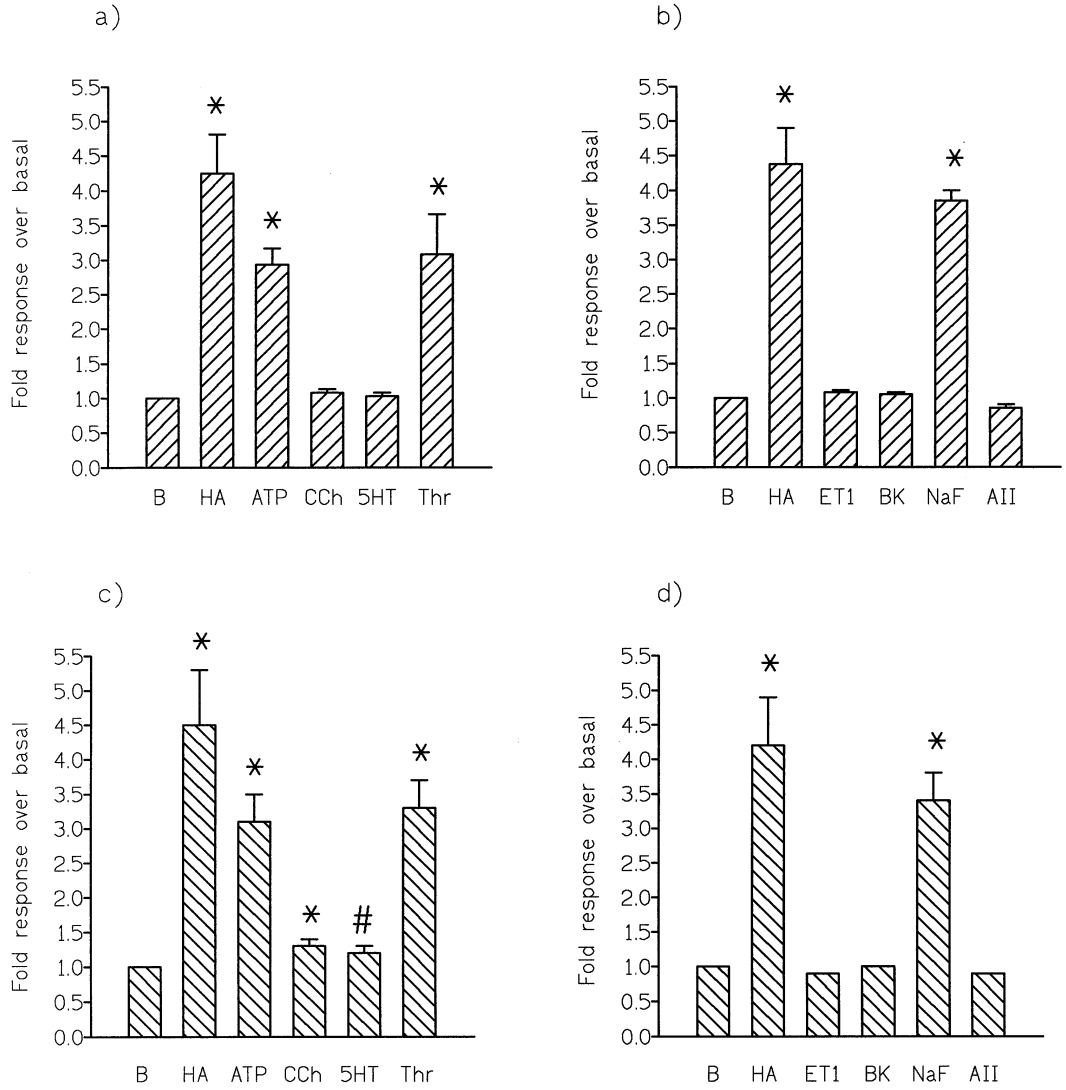

FIG. 3. Accumulation of total [^{3}H]inositol phosphates in response to histamine (100 μM; HA), ATP (1 mM), carbachol (1 mM; CCh), 5-hydroxytryptamine (100 μM; 5-HT), thrombin (1 U/ml; Thr), endothelin-1 (1 μM; ET-1), bradykinin (1 μM; BK), sodium fluoride (20 mM; NaF), angiotensin II (10 μM; AII) or basal levels (B). Results are expressed as the fold response over basal in HUAEC21 (a, b) or HUAEC3 (c, d). Data bars are the mean ± SEM of quadruplicate determinations in four (a, b) or seven (c, d) separate experiments using cells from different donors in each experiment. All cells were at passage 3. The mean basal values were 1715 ± 126 dpm (N = 8; 21% oxygen) and 1287 ± 148 dpm (N = 14; 3% oxygen). *$P < 0.01$; #$P < 0.05$ with respect to basal values (2-way analysis of variance with post hoc Newman–Keuls).

Characteristics of the Histamine-Induced Increase in Phosphoinositide Turnover

The histamine (100 μM)-induced increase in PI turnover in HUAEC3 increased steadily over the time period studied (5–60 min) with a similar time course to that reported previously in cells maintained in a 21% oxygen environment [3]. Histamine (0.1 μM–1 mM) produced a concentration-dependent increase in the accumulation of [^{3}H]-inositol phosphates in both HUAEC3 and HUAEC21 (Fig. 4). The mean log EC_{50} (M) values obtained from these data, −5.11 ± 0.16 (HUAEC3; N = 7) and −5.25 ± 0.11 (HUAEC21; N = 4), were not significantly different. The histamine H_1 receptor antagonist, mepyramine (100 nM) was found to produce parallel shifts of the histamine concentration-response curves to higher concentrations in HUAEC3 (Fig. 4a) and HUAEC21 (Fig. 4b) yielding apparent pA_2 values of 8.39 ± 0.08 (N = 7) and 8.27 ± 0.33 (N = 4) respectively. The apparent pK_D for a series of histamine H_1 receptor antagonists measured in HUAEC3 or HUAEC21 were determined from the inhibition of the [^{3}H]inositol phosphate response to 100 μM histamine (e.g., Fig. 5). These values for each antagonist are similar when comparing the two cell conditions and are not significantly different from each other (Table 2).

[^{3}H]Inositol Phosphate Response to P2-Purinoceptor Agonists

ATP, ATPγS and UTP produced a concentration-dependent increase in the accumulation of [^{3}H]inositol phos-

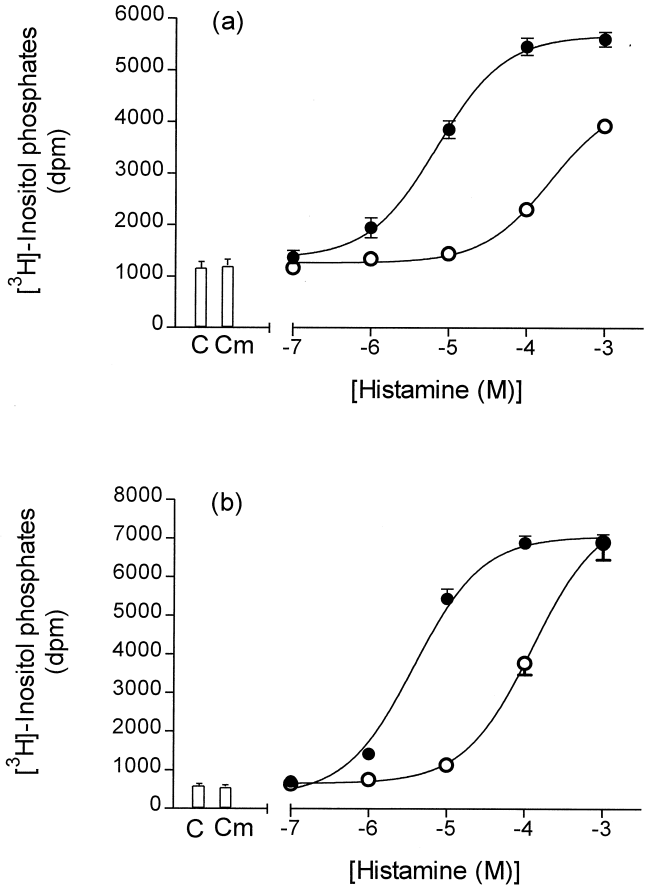

FIG. 4. Accumulation of [^{3}H]inositol phosphates in response to histamine (20 min) in the presence (○) or absence (●) of 100 nM mepyramine in (a) HUAEC3 and (b) HUAEC21. Columns represent basal responses in the absence (C) or presence (Cm) of antagonist. Data points represent the mean of quadruplicate determinations obtained in a single experiment. Bars = SEM. The experiments was repeated 6 (a) or 3 (b) more times using cells from different donors in each with similar results. All cells were at passage 3.

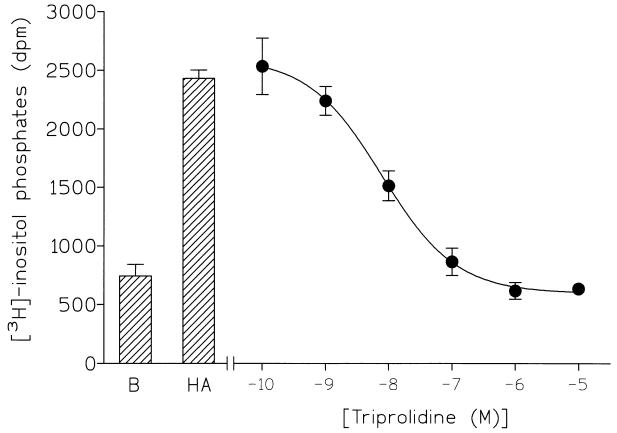

FIG. 5. The effect on the inositol phosphate response to a 20 min incubation of 100 μM histamine produced by triprolidine in HUAEC3. Basal response (B) and the histamine-induced response alone (HA) are represented by the columns. The effect of increasing concentrations of triprolidine added 20 min prior to histamine are shown on the right of the figure. Data points are the means of triplicate determinations and bars show the SEM. The experiment was repeated five more times with similar results using cells from different donors. All cells were at passage 3.

phates in HUAEC21 and HUAEC3 (Fig. 6a and 6b, respectively). The EC_{50} values and the maximum responses (E_{max}) obtained for these agonists did not differ significantly between the two cell types (Table 3). 2-MeS-ATP (100 μM) produced only a weak stimulation of [^{3}H]inositol phosphate accumulation in either HUAEC3 (8.7 ± 4.1% of the response to 0.1 mM histamine; Fig. 6b) or HUAEC21 (E_{max} = 9.5 ± 1.9%; Fig. 6a).

DISCUSSION

This study has demonstrated the ability to isolate human umbilical artery endothelial cells into a 3% oxygen environment and to maintain them in a healthy condition for at least four passages in this atmosphere. HUAEC3 are morphologically similar to HUAEC21 and demonstrate identical growth rates and immunocytochemical staining. Depletion of cellular glutathione levels is a key indicator of free-radical damage to the cell [29], however no depletion of glutathione was noted in HUAEC3 compared to HUAEC21. All the criteria above suggest that cells maintained long term in a 3% oxygen environment remain healthy and viable in that atmosphere.

Oxidative phosphorylation is the primary metabolic pathway used to produce ATP in eukaryotic cells. It has been shown that in cells sensitive to hypoxia, glucose and oxygen consumption are inversely related [30]. Therefore, when the oxygen supply to these cells is limited there is a shift in the metabolic pathway used to produce ATP from oxidative phosphorylation to glycolysis. Indeed studies have demonstrated that there is an increased transcription of genes encoding enzymes for the glycolytic pathway [31]. Furthermore, several studies have shown that endothelial cells demonstrate predominantly a glycolytic pathway for energy production which can be increased in response to hypoxia [5, 32] sufficiently to maintain normal levels of ATP. However, the vast majority of studies of hypoxia have involved exposing cells grown in a 21% oxygen environ-

TABLE 2. Apparent pK_D values for histamine H_1 receptor antagonists in either HUAEC3 or HUAEC21

	Apparent pK_D (M)	
Antagonist	HUAEC21 (N = 5)	HUAEC3 (N = 6)
Chlorcyclizine	9.2 ± 0.4	9.6 ± 0.3
−Chlorpheniramine	5.6 ± 0.3	6.0 ± 0.3
+Chlorpheniramine	8.8 ± 0.3	8.6 ± 0.4
Mepyramine	8.3 ± 0.3	8.4 ± 0.1
Promethazine	10.4 ± 0.5	11.5 ± 0.3
Triprolidine	9.2 ± 0.2	9.1 ± 0.2

Data represent the mean ± SEM in cells from six (HUAEC3) or five (HUAEC21) different donors. Antagonists were incubated for 20 min prior to the addition of 0.1 mM histamine.

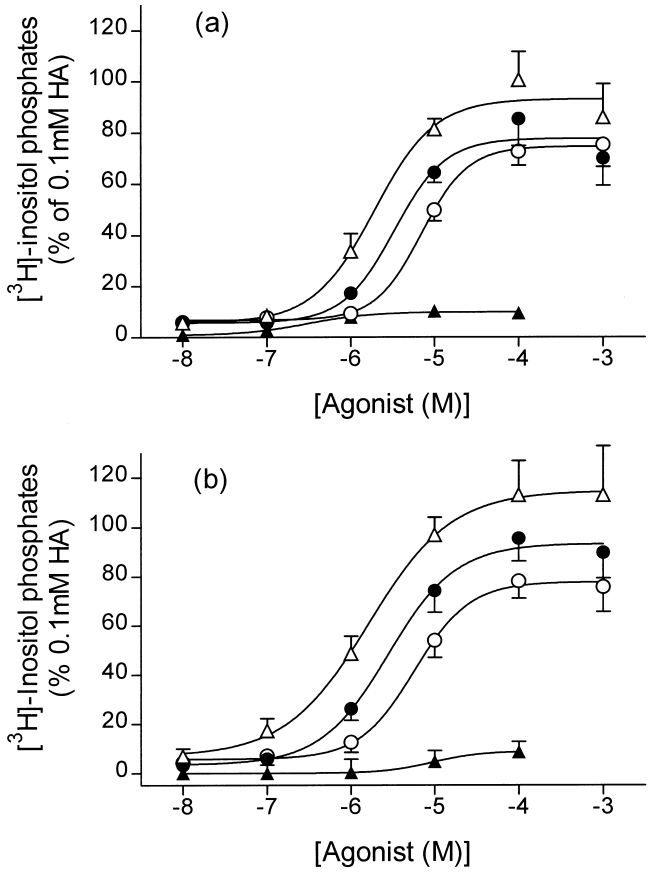

FIG. 6. Total [^{3}H]inositol phosphates accumulated in response to increasing concentrations of UTP (△), ATP (●), ATPγS (○) or 2-methylthioATP (▲) in a) HUAEC21 or b) HUAEC3. Results are expressed as a percentage of the response to 0.1 mM histamine measured in the same experiment. Agonists were incubated with cells for 20 min. Data show mean ± SEM of six separate experiments (each using cells from a different donor). In each experiment triplicate determinations were made at each concentration of agonist. All cells were used at passage 3.

ment to an atmosphere of 0–5% oxygen for periods of up to 48 hr only.

In the present study we found that HUAEC3, grown long term in a low oxygen atmosphere had significantly lower (33%) ATP levels than their HUAEC21 counterparts. In contrast, Tretyakov and Farber [33] have grown BPAEC for up to 4 months in a 3% oxygen environment and found

TABLE 3. Characteristics of the phosphoinositide response of HUAEC21 or HUAEC3 to purinoceptor stimulation

	HUAEC21		HUAEC3	
	$-\text{Log}\ EC_{50}$ (M)	E_{max} Fold over basal	$-\text{Log}\ EC_{50}$ (M)	E_{max} Fold over basal
ATP	5.5 ± 0.1	5.9 ± 0.5	5.5 ± 0.1	4.9 ± 0.6
UTP	5.8 ± 0.2	5.8 ± 0.5	5.8 ± 0.2	4.7 ± 0.4
ATPγS	5.2 ± 0.1	4.9 ± 0.3	5.3 ± 0.1	4.4 ± 0.5

Results are expressed as mean ± SEM in cells from five (HUAEC21) or six (HUAEC3) different donors. Agents were incubated with the cells for 40 min before measurement of total [^{3}H]inositol phosphates as described in "Materials and Methods."

that ATP levels were increased compared to BPAEC grown at 21% oxygen. Explanation of this disparity may lie solely in the difference between the physiological oxygen concentration of the vascular bed of origin of these cells where the pO_2 for BPAEC is approximately 40 mmHg (5%; [33]) and for HUAEC is approximately 16 mmHg (2%; ref. 1) and the oxygen concentration to which they are exposed *in vitro* (3%). Alternatively, the glucose available in the culture medium in the present study (5mM) may not have be sufficient to supply the demand for glycolysis. Nevertheless, despite the decrease in ATP levels at the lower oxygen tension, the levels of ATP found in HUAEC3 appear to be much higher than those obtained in other 'normoxic' (21%) endothelial cells. For example, the ATP content of pig coronary artery endothelial cells and BPAEC grown at 21% was found to be 2 nmol/10^6 cells [12] and 1.3 nmol/10^6 cells [34] respectively. It would appear, therefore, that the level of ATP within a cell depends very much on the cell type.

The level of ATP maintained in HUAEC3 was found to be sufficient to facilitate phosphoinositide (PI) responses to a variety of agonists which were similar to those obtained in HUAEC21. This would indicate that the HUAEC21 maintain an ATP reserve over and above that required for this signaling pathway. Indeed, the $-\log EC_{50}$ values obtained in HUAEC3 to the P_{2y2} (P_{2U})-purinoceptor agonists ATP, UTP and ATPγS are almost identical to those obtained in HUAEC21 and similar to previously reported values [35]. This is further substantiated by the time course of the response to histamine (0.1 mM) in HUAEC3 which is similar to that previously reported by us for HUAEC grown in a 21% oxygen atmosphere [3].

The histamine receptor mediating the PI response in HUAEC3 and HUAEC21 was identified as the H_1 receptor since mepyramine, a H_1-selective antagonist [36] caused a shift of the histamine concentration-response curve to higher concentrations. There was no significant difference between the apparent K_D values obtained in HUAEC3 or HUAEC21 for a range of H_1-receptor antagonists (Table 2) and these were similar to those previously reported in the literature in a number of tissues and species (for review see ref. 37). In addition, K_D values reported from binding data for mepyramine, triprolidine and (+)-chlorpheniramine for the human H_1 receptor clone transfected into CHO cells correlate well with those obtained in the present study [38, 39, 40]. However, of interest are the apparent pK_D values obtained in the present study for the H_1 selective antagonist promethazine, the most potent of the antagonists tested in this study; pK_D (M) = −10.4 in HUAEC21 and −11.5 in HUAEC3. Previous pK_D values quoted in the literature are 8.9 in guinea-pig brain and 8.7 in the rat brain [37]. It is possible that these differences in pK_D values may reflect subtle differences in the genetic coding of the histamine H_1 receptor between species.

In summary, this study has demonstrated that human umbilical artery endothelial cells can be grown long term in a low oxygen environment without any detrimental effect

on their phosphoinositide signalling in response to a variety of agonists. Further studies can now extend to establishing whether changes occur in the signalling mechanisms of the HUAEC3 cells when they are exposed, short term, to an increase in oxygen tension thereby simulating the circulatory changes occurring in the umbilical artery after delivery.

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References

1. Wulf H, The oxygen and carbon dioxide tension gradients in the human placenta at term. *Am J Obstet Gynaecol* **88:** 38–44, 1966.
2. MacLennan SJ, McGrath JC and Whittle MJ, Inhibition of the oxygen-induced contraction of the isolated human umbilical artery by indomethacin, florbiprofen, aspirin and drugs modifying Ca^{2+} disposition. *Prostaglandins* **36:** 711–729, 1988.
3. Hawley J, Rubin PC and Hill SJ, Distribution of receptors mediating phosphoinositide hydrolysis in cultured human umbilical artery smooth muscle and endothelial cells. *Biochem Pharmacol* **49:** 1005–1011, 1995.
4. Jaffe EA, Cell biology of endothelial cells. *Hum Pathol* **18:** 234–239, 1987.
5. Loike JD, Cao L, Brett J, Ogawa S, Silverstein SC and Stern D, Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol* **263:** C326–C333, 1992.
6. Kourembanas S, Hannan RI and Faller DV, Oxygen tension regulates the expression of the platelet-derived growth factor-B chain in human endothelial cells. *J Clin Invest* **86:** 670–674, 1990.
7. Kourembanas S, Marsden PA, McQuillan LP and Faller DV, Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest* **88:** 1054–1057, 1991.
8. Liu Y, Cox SR, Morita T and Kourembanas S, Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. *Circ Res* **77:** 638–643, 1995.
9. Liao JK, Zulueta JJ, Yu F-S, Peng H-B, Cote CG and Hassoun PM, Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. *J Clin Invest* **96:** 2661–2666, 1995.
10. Zimmerman LH, Levine RA and Farber HW, Hypoxia induces a specific set of stress proteins in cultured endothelial cells. *J Clin Invest* **87:** 908–914, 1991.
11. Graven KK, Troxler RF, Kornfield H, Panchenko MV and Farber HF, Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J Biol Chem* **269:** 24446–24453, 1994.
12. Bhat GB and Block ER, Effect of hypoxia on phospholipid metabolism in porcine pulmonary artery endothelial cells. *Am J Physiol* **262:** L606–L613, 1992.
13. Lee S-L and Fanburg BL, Serotonin uptake by bovine pulmonary artery endothelial cells in culture. II. Stimulation by hypoxia. *Am J Physiol* **250:** C766–C770, 1986.
14. Farber HW and Barnett HF, Differences in prostaglandin metabolism in cultured aortic and pulmonary arterial endothelial cells exposed to acute and chronic hypoxia. *Circ Res* **68:** 1446–1457, 1991.
15. Tretyakov AV and Farber HW, Endothelial cell phospholipid distribution and phospholipase activity during acute and chronic hypoxia. *Am J Physiol* **265:** C770–C780, 1993.
16. Reilly FD and Russe PT, Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. *Anat Rec* **188:** 277, 1977.
17. Walker DW and Mclean JR, Absence of adrenergic nerves in human placenta. *Nature* **229:** 344–345, 1971.
18. Resink TJ, Grigorian GY, Moldabaeva AK, Danilov SM and Buhler FR, Histamine-induced phosphoinositide metabolism in cultured human umbilical vein endothelial cells: association with thromboxane and prostacyclin release. *Biochem Biophys Res Commun* **144:** 438–446, 1987.
19. Chaudhuri G, Buga GM, Gold ME, Wood KS and Ignarro LJ, Characterisation and actions of human umbilical endothelium-derived relaxing factor. *Br J Pharmacol* **102:** 331–336, 1991.
20. Pollock WK, Wreggett KA and Irvine RF, Inositol phosphate production and Ca^{2-} mobilisation in human umbilical vein endothelial cells stimulated by thrombin and histamine. *Biochem J* **256:** 371–376, 1988.
21. Carter TD, Hallam TJ, Cusack NJ and Pearson JD, Regulation of P2y-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br J Pharmacol* **95:** 1181–1190, 1988.
22. Hallam TJ, Pearson JD and Needham LA, Thrombin-stimulated elevation of human endothelial cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochem J* **251:** 243–249, 1988.
23. Semenza GL, Roth PH, Fang H-M and Wang GL, Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* **269:** 23757–23763, 1994.
24. Karsten U and Wollenberger A, Determination of DNA and RNA in homogenised cells and tissues by surface fluorimetry. *Anal Biochem* **46:** 135–148, 1972.
25. Saville B, A scheme for the colourimetric determination of microgram amounts of thiols. *Analys* **83:** 670–672, 1958.
26. Hall IP and Hill SJ, β_2-Adrenoceptor stimulation inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br J Pharmacol* **95:** 1204–1212, 1988.
27. Lazareno S and Roberts FF, Measuring muscarinic antagonist potency using phosphoinositide breakdown in rat cortex slices. *Br J Pharmacol* **92:** 677P, 1987.
28. Darnell J, Lodish H and Baltimore D, *Molecular Cell Biology*, p. 137. New York, Scientific American Books, 1986.
29. Berry MN, Edwards AM and Barritt GJ, *Isolated Hepatocytes: Preparation. Properties and Applications*. p. 195. Amsterdam, Elsevier Science Publishers, 1991.
30. Hochachka PW, Defense strategies against hypoxia and hypothermia. *Science* **231:** 234–241, 1986.
31. Webster KA, Gunning P, Hardeman E, Wallace DC and Kedes L, Coordinate reciprocal trends in glycolytic and mitochondrial transcript accumulations during the in vitro differentiation of human myoblasts. *J Cell Physiol* **142:** 566–573, 1990.
32. Mertens S, Noll T, Spahr R, Krutzfeldt A and Piper HM, Energetic response of coronary endothelial cells to hypoxia. *Am J Physiol* **258:** H689–H694, 1990.
33. Tretyakov AV and Farber HW, Endothelial cell tolerance to hypoxia. Potential role of purine nucleotide phosphates. *J Clin Invest* **95:** 738–744, 1995.
34. Lee S-L and Fanburg BL, Glycolytic activity and enhancement of serotonin uptake by endothelial cells exposed to hypoxia/anoxia. *Circ Res* **60:** 563–658, 1987.
35. Megson AC, Dickenson JM, Townsend-Nicholson A and Hill SJ, Synergy between the inositol phosphate responses to transfected human adenosine A_1-receptors and constitutive P_2 purinoceptor in CHO-K1 cells. *Br J Pharmacol* **115:** 1415–1424, 1995.

36. Adams HM and Hye HKA, Concentration of histamine in different parts of brain and hypophysis of cat and its modification by drugs. *Br J Pharmacol* **28:** 137–152, 1966.
37. Hill SJ, Distribution, properties and functional characteristics of three classes of histamine receptor. *Pharmacol Revs* **42:** 45–83, 1990.
38. DeBacker MD, Gommeren W, Moereels H, Nobels G, van Gompel P, Leysen JE and Luyten WHM, Genomic cloning, heterologous expression and pharmacological characterisation of a human histamine H_1 receptor. *Biochem Biophys Res Commun* **197:** 1601–1608, 1993.
39. Moguilevsky N, Varsalona F, Noyer M, Gillard M, Guillaume JP, Garcial I, Szpirer C, Bollen A and Szpirer J, Stable expression of human H_1 histamine-receptor cDNA in chinese hamster ovary cells—Pharmacological characterisation of the protein, tissue distribution of messenger RNA and chromosomal localisation of the gene. *Eur J Biochem* **224:** 489–495, 1994.
40. Smit MJ, Timmerman HG, Hijzelendoorn JC, Fukui H and Leurs R, Regulation of the histamine H_1 receptor stably expressed in Chinese hamster ovary cells. *Br J Pharmacol* **117:** 1071–1080, 1996.