Extracellular ATP-mediated Signaling for Survival in Hyperoxia-induced Oxidative Stress*

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Respiratory failure is a serious consequence of lung cell injury caused by treatment with high inhaled oxygen concentrations. Human lung microvascular endothelial cells (HLMVEC) are a principal target of hyperoxic injury (hyperoxia). Cell stress can cause release of ATP, and this extracellular nucleotide can activate purinoreceptors and mediate responses essential for survival. In this investigation, exposure of endothelial cells to an oxidative stress, hyperoxia, caused rapid but transient ATP release (20.03 ± 2.00 nM/10⁶ cells in 95% O₂ versus 0.08 ± 0.01 nM/10⁶ cells in 21% O₂ at 30 min) into the extracellular milieu without a concomitant change in intracellular ATP. Endogenously produced extracellular ATP-enhanced mTOR-dependent uptake of glucose (3467 ± 102 cpm/mg protein in 95% oxygen versus 2100 ± 112 cpm/mg protein in control). Extracellular addition of ATP-activated important cell survival proteins like PI3-kinase and extracellular-regulated kinase (ERK-1/2). These events were mediated primarily by P₂Y receptors, specifically the P₂Y₂ and/or P₂Y₆ subclass of receptors. Extracellular ATP was required for the survival of HLMVEC in hyperoxia (55 ± 10% surviving cells with extracellular ATP scavengers [apyrase + adenosine deaminase] versus 95 ± 12% surviving cells without ATP scavengers at 4 d of hyperoxia). Incubation with ATP scavengers abolished ATP-dependent ERK phosphorylation stimulated by hyperoxia. Further, ERK activation also was found to be important for cell survival in hyperoxia, as treatment with PD98059 enhanced hyperoxia-mediated cell death. These findings demonstrate that ATP release and subsequent ATP-mediated signaling events are vital for survival of HLMVEC in hyperoxia.

Cells release ATP either under basal conditions or in response to stress or certain stimuli (1, 2). Mechanical stress, hypotonic media, vasoactive agents, inflammation, cAMP and ATP itself can stimulate ATP release from cells (3). Extracellular ATP binds to specific plasma membrane receptors called P₂ receptors. Depending on the cell type, binding of ATP to its receptors can initiate important signaling events like (i) increases in intracellular calcium; (ii) activation of phospholipases; (iii) modulation of cell volume; (iv) inhibition of platelet aggregation; (v) alteration of vascular tone; (vi) neurotransmission; (vii) elevation of cardiac/skeletal muscle contractility; (viii) immune cell activation; (ix) increased ciliary beat frequency and mucus secretion; and (x) enhanced cell growth and proliferation (3). Although routes of ATP release are not well defined, cytolysis, vesicle-mediated ATP release, and ATP release via channels like ATP-binding cassette (ABC) proteins and connexin hemichannels may contribute (1, 4).

The role of ATP release in oxidative stress is not clear. Severe oxidative stress can diminish intracellular ATP (5, 6). Delayed catabolism of extracellular nucleotides due to diminished ectonucleotidase activity in the presence of reactive oxygen species (ROS) also has been suggested (7). Whether extracellular ATP release occurs due to oxidative stresses like hyperoxia, and what the functional significance of this process could be is unknown. Microvascular endothelial cells are primary targets of hyperoxic lung injury (8) and in other forms of acute respiratory failure. Hypoxic preexposure of human lung microvascular endothelial cells (HLMVEC) attenuates cell death caused by subsequent hyperoxic exposure (9). This is partially attributed to up-regulation of PI 3-kinase (PI3K), a component of an established cell survival pathway. Previously we showed that hypoxic (1 or 3% O₂) exposure induces ATP release in HLMVEC and fetal lung fibroblasts (10). There, hypoxia and ATP act synergistically to upregulate common proliferation-inducing pathways in fibroblasts. Extracellular ATP and purinergic receptor activation play a critical role in determining the intracellular effects of key growth-regulating factors (11–13). Since hypoxic conditions favor proliferation and ATP release in endothelial cells (14, 15), we speculated that ATP release, if present in hyperoxia, could activate important survival pathways. Using primary HLMVEC, we demonstrate that ATP is released in hyperoxia and that the presence of extracellular ATP is essential for survival of these cells. We determined the effect of extracellular ATP on glucose uptake, PI3K activity, p70 S6 kinase phosphorylation, and ERK-1/2 phosphorylation in HLMVEC. Our findings strongly support the notion that extracellular ATP, through purinergic receptors, signals these important events.

EXPERIMENTAL PROCEDURES

Cells and Culture—Human lung microvascular endothelial cells (HLMVEC) were purchased from Clonetics Ltd (San Diego, CA) and

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‡§ The abbreviations used are: ROS, reactive oxygen species; ADA, adenosine deaminase; HLMVEC, human lung microvascular endothelial cells; ERK-1/2, extracellular-regulated kinase p44 and p42; mTOR, mammalian target of rapamycin; cPLA₂, cytoplasmic phospholipase A₂; A₅₃, adenosine 5′-O-(thiodiposphate); ANOVA, analysis of variance; DOG, 2-deoxylucose; MeSO₂, dimethyl sulfoxide.
cultured as per the manufacturer’s protocol in 100-mm dishes (9). Hyperoxic exposures were performed at sea level atmospheric pressure as described previously (16, 17). The cells were 70–80% confluent when exposed. These cells growth arrest within 24 h in hyperoxia and, thus, cultures never exceeded 90% confluence. In order to match confluence of cells, in normoxia (21% O2) were studied after 24 h in culture. Fresh medium was supplied daily during hyperoxic exposures.

**Determination of Extracellular and Intracellular ATP Content**—Total ATP content in extracellular medium was detected with a luciferase-luciferin kit (Analytical Luminescence Laboratory, Sparks, MD) using a Monolight 3010 luminometer (Analytical Luminescence Laboratory) (10). Cells in 100-mm cell culture dishes were exposed to either normoxia (21% O2, 5% CO2, balance N2) or hyperoxia (95% O2, 5% CO2) at 5% CO2 full flow rate at 37 °C for the indicated time periods. At the beginning of the experiment, medium was drained from cells and fresh medium that had been pre-equilibrated with the appropriate gas mixture was infused onto the cell monolayer. After incubation, 1 ml of conditioned medium was collected into chilled polypropylene tubes (Sigma) and centrifuged at 12,000 g for 10 min to remove any cell debris. Individual 100-µl aliquots were taken and the luciferin-luciferase assay was carried out as described previously (18).

2-[3H]Deoxyglucose Uptake—2-Deoxyglucose (DOG) uptake was determined as described (19). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum or maintained in glucose-free DMEM in 6-well plates 24 h prior to experiments. For measurement of 2-[3H]deoxyglucose uptake, cells were washed twice with Krebs-Ringer-phosphate-HEPES (KRP-HEPES) buffer (25 mM HEPES, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1.2 mM KH2PO4, 2.5 mM MgSO4, 5 mM NaHCO3, and 0.1% bovine serum albumin) and incubated in 950 µl of KRP-HEPES buffer for 60 min. Cells were then treated with ATP for 30 min or exposed to hyperoxia for 4 h. Glucose uptake was measured by adding 20 µl of glucose mixture (5 mM 2-deoxyglucose, 0.5 µCi of [3H]deoxyglucose in KRP-HEPES buffer) to 980 µl of KRP-HEPES buffer and incubating for 20 min at 37 °C. Nonspecific glucose uptake was measured in parallel incubations in the presence of 10 mM cytochalasin B, which blocks transporter-mediated glucose uptake, and was subtracted from total uptake in each assay. Uptake was terminated by washing the cells three times with 1 ml of ice-cold phosphate-buffered saline. Cells were subsequently lysed with 0.5 ml of 0.5 M NaOH solution containing 0.1% SDS, and the solution was rotated for 15 min. Cell-associated radioactivity was measured by a liquid scintillation counter.

**Assay for PI 3-Kinase Activity**—PI3K activity was assessed as described previously by the incorporation of [32P]ATP to exogenous phosphoinositide resulting in the production of PI 3-phosphate (PI3P) (9).

**Western Blot Analysis of Cellular Extracts for Phospho-ERK-1/2 and Phospho-p70 S6 Kinase**—Cells were harvested and Western blot analysis was performed for the detection of phospho-ERK and total ERK as previously reported (10). Antibodies against phosphorylated p70 S6 kinase were obtained from Upstate Biotechnology (Lake Placid, NY) and used at a dilution of 1:1000.

**Measurement of Arachidonic Acid (AA) Release**—The protocol used for determining AA release was as described (20). HLMVEC were plated in 6-well plates at 60,000 cells/well and incubated in growth medium until they reached 60–70% confluence. Cells were then washed twice with serum-free medium and incubated with 0.5 µCi of [3H]AA/well in serum-free medium overnight. Cells were then washed to remove unincorporated [3H]AA and incubated in HBSS supplemented with 0.65% BSA. Hyperoxic exposure or incubation with ATP was performed, and the medium was collected at appropriate times. Medium was centrifuged at 500 g for 5 min, and the amount of radioactivity in the supernatant was determined by scintillation counting. Cells were scraped in 0.5 ml of 0.1% Triton X-100 for determining the total cellular radioactivity.

**Detection of Prostaglandins**—After exposure of HLMVEC to hyperoxia for the specified duration, medium was collected and centrifuged at 100,000 g, and supernatants were collected and frozen at −70 °C. Prostaglandins pGE2 and 6-keto-pGF1α were analyzed using an ELISA kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer’s instructions.

**Detection of Cell Survival and Protein Concentration**—Cell death in HLMVEC was assessed by using YOYO-1 (Molecular Probes, Eugene, OR), a plasma membrane impermeable dye that binds irreversibly to DNA (21) as described before (9). Protein concentration in cell lysates was determined using the BioRad DC protein assay kit in a 96-well plate with bovine serum albumin as a standard.

**Statistical Analysis**—All statistical calculations were performed with JMP and SAS software (SAS Institute, Cary, NC). Means were compared either by two-tailed Student’s t test for comparison between two groups or one-way analyses of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons of analyses involving three or more groups. A p value of < 0.05 was considered significant. For 2-deoxyglucose uptake experiments 2-way ANOVA was performed. Dunnett’s test was used to compare levels of a factor (treatment or inhibitor) to controls within each level of the factor. This procedure was applied only if a significant interaction was observed for the 2-way ANOVA. A p value of < 0.01 was considered significant.

**RESULTS**

**Effect of Hyperoxia on Extracellular ATP Concentration in HLMVEC**—Exposure of human lung microvascular endothelial cells to hyperoxia (95% O2) elicited an increase in extracellular ATP in a time-dependent manner (10–120 min) (Fig. 1). The experiment was performed both with and without serum to exclude any possible contribution of growth factors (medium without serum had no growth factors added) or serum. Extracellular ATP at 10 min was 0.07 ± 0.01 nmol/106 cells in 21% O2 and 0.33 ± 0.02 nmol/106 cells in 95% O2 in presence of serum (Fig. 1). Accumulation of ATP in the extracellular medium was maximal at 30 min, being 20.03 ± 2.00 nmol/106 cells in 95% O2 as compared with 0.08 ± 0.01 nmol/106 cells in 21% O2. At 60 and 120 min, extracellular ATP had diminished and at 4 and 24 h, returned to control levels (Fig. 1). There was no significant change in total intracellular ATP levels during hyperoxic exposure at these times (inset, Fig. 1).

**Role of Extracellular ATP in HLMVEC Survival in Hyperoxia**—Although extracellular ATP can stimulate proliferation in many cell types, including endothelial cells (10, 22), little is known about its role in modulating survival of cells in hyperoxia. To determine this, cells were treated with etonucleotide (a mixture of apyrase grades VI and VII, Sigma) and adenosine deaminase (ADA). Actions of both enzymes can eliminate extracellular ATP and its degradation product adenosine (2). Cells exposed to hyperoxia (4 days) in the presence of apyrase (2 units/ml) had a significant decrease in viability compared with untreated, hyperoxia-exposed cells (Fig. 2). A nonsignificant further decline in surviving cells was observed when ADA (2 units/ml) was included with apyrase (Fig. 2).

**Effect of Extracellular ATP on Glucose Metabolism in HLMVEC**—Hyperoxia increases glucose consumption in human lung epithelial-like A549 (18, 23) and other cells. Results obtained in the same cells indicated enhanced preservation of intracellular ATP with frequent glucose supplementation in hyperoxic conditions (Fig. 3).
HLMVEC during hyperoxic exposure, cells were treated with extracellular ATP, via modulation of mTOR, affects survival of Mammalian TOR is a central regulatory element of metabolic (24). ATP-induced phosphorylation was sensitive to rapamycin, was observed upon ATP treatment of HLMVEC (Fig. 3, A time-dependent increase in phosphorylation of p70 S6 kinase prompted us to determine the effect of ATP on p70 S6 kinase. inhibition of ATP-induced glucose uptake by rapamycin and glutamine metabolism are critical in cells for protection others (16, 18, 25) have demonstrated that increased glucose zation (24). A substantial increase in lung RNA and protein Mammalian TOR is an important regu-

Effect of Rapamycin on Survival of HLMVEC in Hyperoxia—Mammalian TOR is a central regulatory element of metabolic state adjustment for survival under stress (26). To determine if extracellular ATP, via modulation of mTOR, affects survival of HLMVEC during hyperoxic exposure, cells were treated with rapamycin (50 and 100 nm) for 30 min and then exposed to hyperoxia (95% O₂, 5% O₂) at sea level pressure for 6 days. Appropriate controls having equal dilutions of dimethyl sulfoxide, solvent vehicle for rapamycin, also were included. Fresh media were supplied daily. After exposure, cell death was determined using YOYO-1 staining. Cell death was 24 ± 4% at 6 days of hyperoxic exposure in untreated HLMVEC (Fig. 4). Cell death was similar in cells treated with Me₂SO prior to exposure. Treatment with rapamycin (50 nm) along with hyperoxia resulted in 31 ± 8% cell death. A further increase was observed when cells were treated with a higher concentration of rapamycin (40 ± 8% in hyperoxia versus 13 ± 4% in normoxia at 100 nM rapamycin). These data, reflecting the percentage of surviving cells among those which remained adherent after hyperoxic exposure, indicate that a rapamycin-sensitive signal is important in cell survival under these conditions.

Effect of Extracellular ATP on PI 3-Kinase Activity—PI 3-kinase (PI3K) plays a critical role in survival of HLMVEC in hyperoxia (9). It is also implicated in insulin-stimulated glucose transport (27). ATP-dependent glucose transport also was found to be inhibitable by LY294002, a PI3K inhibitor (28). Therefore, we examined possible involvement of this pathway in HLMVEC in relation to stimulation by extracellular ATP. PI3K activity was increased by more than ~2-fold after treatment with ATP for 5 min. Activity was maximal at 15 min, but additional incubation time did not further increase PI3K activity (Fig. 5, upper panel). Interestingly, various analogs of ATP, ADP, AMP, and adenosine also were effective in increasing PI3K activity (Fig. 5, lower panel). Adenosine, AMP, and UDP were most effective, causing ~3–4-fold increases. ATP, ADP, and their stable analogs ATP-S and ADP-S increased PI3K activity ~2-fold. The ATP analog β-methyl ATP was not effective, whereas 2-methyl-thio-ATP also enhanced PI3K activity >2-fold. Cholera toxin, an inhibitor of small G protein G_s, was not effective in diminishing the ATP-induced PI3K activity (Fig. 5, lower panel). Hyperoxia is an established stimulus of PI3K in HLMVEC, and LY294002 inhibits it in this context. Hence, these stimuli were used as controls (9).

Effect of ATP and Oxygen on Early Phosphorylation of ERK—P2 receptors stimulate ERK-1/2 (10, 29), and ERK-1/2 also mediate glucose uptake by insulin, growth factors or other stimuli (30–32). HLMVEC were exposed to hyperoxia (95% O₂) for 30, 60, and 120 min with and without serum. Phosphorylation of both ERK-1 and ERK-2 by hyperoxia was observed at 30 (Fig. 6A). Significantly increased ERK phosphorylation was detected at 60 ‘but not at 120’. At later times (16 and 24 h) hyperoxia did not increase ERK phosphorylation (data not shown), nor did hyperoxic exposure in the absence of serum cause this effect.

Extracellularly added ATP (100 μM) induced ERK-2 phosphorylation within 2 min (Fig. 6B). ERK-2 phosphorylation was maximal at 5 min, and at 15 min both ERK-1 and ERK-2 were phosphorylated. Treatment of cells with ATP in the presence of its scavenger apyrase, or a mixture of apyrase and ADA, abolished ATP-dependent ERK phosphorylation (Fig. 6C). In addition, apyrase plus ADA markedly inhibited ERK-1/2 phosphorylation induced by hyperoxia, whereas either component alone, apyrase or ADA, was less effective (not shown). These data indicate that endogenously produced nucleotides are important in ERK signaling in hyperoxia.

Effect of P2 Receptors on ATP-induced ERK Phosphorylation—ATP induces MEK-specific ERK-1/2 phosphorylation as ATP-dependent ERK phosphorylation was inhibited by PD98059, a specific extracellular-regulated kinase kinase (MEK-1/2) inhibitor (Fig. 7A). Suramin, a nonspecific P2 receptor antagonist, prevented ERK phosphorylation by ATP. ERK phosphorylation was also abolished by a P₂Y receptor antagonist, Cibacron blue. ERK phosphorylation by extracellular ATP was 50% inhibited by PPADS, a less selective P₂X antagonist, which can inhibit some P₂Y receptors (33). Pertussis toxin (100
FIG. 3. Effect of extracellular ATP on phosphorylation of p70 S6 kinase, a target of mTOR. Serum-starved HLMVECs were incubated with ATP (100 μM) for 0–15 min (panel A). Whole cell extracts were probed by Western blot using antibodies specific for phosphorylated p70 S6K. Cells also were preincubated with 100 nM rapamycin for 30 min before stimulation with ATP for 15 min (panel B). Results were reproduced in three separate experiments.

ng/ml), an inhibitor of G proteins, did not attenuate activation ERK by ATP.

ATP, its stable analog ATPγS, and UDP were most effective in inducing ERK-1/2 phosphorylation (–4–5-fold) (Fig. 7B). Adenosine, ATP, and its stable analog ATP/βS increased ERK-1/2 phosphorylation –3-fold. A –2-fold increase in ERK activity resulted upon incubation with UDP. Furthermore, 2-methyl-thio-ATP, an effective P2Y1 agonist, and βγ-methyl ATP, had no significant effect on ERK-1/2 phosphorylation. ATP-dependent ERK phosphorylation also was not affected by cholina toxin. Collectively these results indicate the potential involvement of P2Y1 receptors in ATP-dependent ERK phosphorylation, and support potential roles of ATP and adenosine, as suggested by the effects of apyrase plus ADA in hyperoxia.

Effect of MEK Inhibition by PD98059 on Survival of HLMVEC in Hyperoxia—Hyperoxic exposure induced ATP release in HLMVEC and extracellular ATP enhanced ERK phosphorylation. In addition, increased ATP phosphorylation resulted from hyperoxic exposure. Hence, ERK phosphorylation could be of significance in survival of HLMVEC in hyperoxia. We determined the extent of cell death with and without a specific inhibitor of MEK, PD98059, in hyperoxia for 6 days. For inhibitor treatments, cells were preincubated with PD98059 (10 μM) for 30 min and then exposed to hyperoxia, including the inhibitor for either 24 h or 6 days of hyperoxia. No significant difference in cell death was observed between cells treated for 24 h with PD98059 in 21% oxygen and the solvent-treated control cells. By contrast, cells exposed to hyperoxia and PD98059 (24 h) did show increased death (Fig. 8). Cells treated for 6 days with PD98059 exhibited a small increase in cell death in normoxia (21 ± 4 versus 14 ± 2%), which increased to 50% in cells treated with PD98059 throughout the 6-day hyperoxic exposure (Fig. 8).

We also determined the effect of treatment with the inhibitor PD98059 on ERK phosphorylation due to ATP in HLMVEC. Treatment for 24 h with PD98059 (10 μM) inhibited ATP-dependent ERK phosphorylation reversibly in HLMVEC. Adding fresh medium with ATP without the MEK inhibitor caused enhanced ERK phosphorylation at 15 min in both control (lane 2, Fig. 9) and 24 h inhibitor-treated HLMVEC (ATP treatment after removing the inhibitor, lane 3, Fig. 9). As further assessment of inhibitor stability, the inhibitor was found to be active at 24 h in the medium, as no increased ERK phosphorylation was observed in cells in which ATP was added directly to media containing inhibitors at that time (no fresh medium, lane 4, Fig. 9). Taken together, these data indicate that extracellular ATP causes ERK phosphorylation through MEK and that this process is critical in determining cell survival in hyperoxia.

Effect of Extracellular ATP or Hyperoxia on AA Release

Effect of Extracellular ATP on AA Release—Intracellular arachidonic acid (AA) provides an important immediate source of the eicosanoids, the most abundant class of lipid mediators. It is important to determine if ATP-induced effects on AA release are occurring in a manner similar to that observed with hypoxia. We made use of a commercial kit that uses [3H]arachidonic acid labeled in the 5α-position to measure extracellular [3H]arachidonic acid at baseline and after treatment with ATP or hypoxia. We found that ATP and hypoxia both caused an increase in extracellular AA release in HLMVEC (Fig. 10). The magnitude of AA release was greater with ATP than hypoxia, and both treatments caused the same magnitude of AA release.

FIG. 4. Effect of rapamycin on cell death of HLMVEC in hyperoxia. Rapamycin (50 and 100 nM) was added to medium, and cells were exposed to hyperoxia after 30 min. Medium was changed daily and fresh inhibitor was added. Results are mean ± S.E. (*, p < 0.05 compared with untreated, hyperoxia-exposed cells; #, p < 0.05 compared with rapamycin-treated, normoxic cells). Similar results were found in three experiments.
Hyperoxia Causes ATP Release—This investigation demonstrates ATP release due to an oxidative stress, exposure to hyperoxia. Other stresses, such as shear, hypotonia, and hyperoxia, also can cause ATP release (10, 12, 38, 39). Here, ATP was released and accumulated in extracellular medium for up to 2 h, but then decreased, potentially because of ectonucleotidases and/or receptor binding and internalization. Endothelial cells possess ecto-ATPase activity, which degrades extracellular ATP (40). In our experiments, extracellular ATP accumulated despite such activity. Importantly, there was considerably less effect on extracellular ATP by ectonucleotidase inhibition than by exposure to hyperoxia, suggesting that high oxygen concentrations mediate direct ATP release by endothelial cells.

Relative to ATP release, enhanced membrane fluidity and damage in endothelial cells occur at later time points in hyperoxia (2 days) (41), and biochemical, mitochondrial, plasma membrane, and cellular injuries are time dependent in vitro and in vivo (10, 16, 22, 42). In fact, cell death due to hyperoxia requires at least 6–8 days in cultured human lung microvascular endothelial cells (9). By contrast, ATP release is a very early event, which declines during prolonged exposure. Hence, cell damage is unlikely cause of ATP release upon acute exposure to hyperoxia. In this study, total intracellular ATP content remained unchanged during this time. Cell ATP normally is preserved during early hyperoxic exposure provided sufficient glucose (18) and glutamine (16) are available. However, the decline in ATP release could be due to depletion of specific pools of ATP utilized for this purpose and/or declining cellular respiratory capacity (42).

Extracellular ATP is a potent autocrine and paracrine signal modulating cellular functions through activation of P2-purinoceptors (2). Schwert et al. (13) found that various endothelial cells including HLMVEC contain P_{2Y} (P_{2Y2}) and multiple P_{2X} receptors on their surface. They also described endothelial cells as a rich source of ATP, released both under basal and stimulated conditions (13), and mediating autocrine and/or paracrine signaling events in cells that also express purinergic receptors. ATP released from nerve cells and endothelial cells may modulate vascular tone (43). In addition, extracellular ATP may influence migration, proliferation and death of vascular smooth muscle cells and endothelial cells (44). Activation of P_{2Y} and P_{2Y2}, along with adenosine A2 receptors, also is linked to MAPK-dependent regulation of smooth muscle, lung epithelial, and endothelial cell proliferation (45, 46, 10).

Endothelial cells are primary targets of hyperoxic damage in lungs (8, 9). In this study, hyperoxia-induced endothelial cell death was accelerated by the ectonucleotidase, apyrase, which degrades ATP, indicating that extracellular ATP is important in regulating survival. The degradation products of ATP or adenosine are not toxic to pulmonary endothelial cells (47) indicating that the enhanced cell death observed was due to depletion of extracellular nucleotides. A network of ectoenzymes including ectonucleotidase and ADA is expressed both by endothelial and hematopoietic cells, which tightly regulate extracellular nucleotides (12, 48) and whose existence makes the magnitude of the ATP signal found here even more remarkable.

To our knowledge, this is the first report indicating a role for...
extracellular ATP in survival and adaptation of cells during oxidative stresses like hyperoxia. Increased blood plasma ATP levels in the pulmonary vasculature of hyperoxia-exposed fetal lambs has been demonstrated indicating that the current observations in cell culture could have in vivo relevance (49). In juxtaposition to the present study, extracellular ATP and its breakdown products at high concentrations may have deleterious effects on endothelial cell survival. Prolonged incubation (24 h) of human pulmonary artery endothelial cells with high concentrations of ATP (100 μM) and adenosine in HEPES buffer caused apoptotic cell death (50). However, incubation with very high ATP concentrations (500 μM) in bovine pulmonary artery endothelial cells growing in complete medium (containing serum) did not cause injury (47), indicating the critical importance of culture conditions. In the present study, ATP was released in nanomolar quantities and was detectably elevated for a relatively short interval.

Hyperoxia Activates mTOR: Increased Glucose Utilization and Survival — Enhanced glucose utilization is an important feature of hyperoxic adaptation which supports cell ATP and survival (18). Extracellular addition of ATP (100 μM) causes enhanced expression of glucose transporter GLUT-1 and increased cellular glucose uptake (28). Our results indicate that extracellular ATP and hyperoxia increase P2 receptor-dependent glucose uptake in HLMVEC. Further, both ATP- and hyperoxia-mediated enhanced glucose uptake were sensitive to rapamycin. Mammalian TOR is a homeostatic energy sensor (26) and important mediator of growth and proliferation in vascular cells (24, 51). Moreover, extracellular ATP-mediated phosphorylation of p70 S6 kinase, a downstream effector of mTOR, occurs in other cell types (51). In this study, extracellular ATP induced mTOR-specific phosphorylation of p70 S6 kinase. In addition, enhanced cell death observed in the presence of rapamycin in hyperoxia indicated that mTOR-dependent signaling is critical for survival.
Cell death due to mTOR inhibition was greater than in controls only after 6 days of hyperoxia, whereas cell death due to ATP scavenging began within 4 days. Thus, other ATP-dependent signaling pathways could be important. PI3K is an established survival signal protein (45, 51), and ATP-dependent enhanced PI3K activity has been reported (28, 45). Extracellular ATP enhanced PI3K activity in HLMVEC in a time-dependent manner. Enhanced PI3K activity was due to P2Y receptor activation, as indicated by the activity profile of ATP analogs, although adenosine also could effect this action. PI3K may maintain cell size and survival, at least in part, by increasing mTOR-dependent glucose uptake (51). In hyperoxia, PI3K function could be both mTOR-dependent and -independent.

**ATP Activates ERK and Signals Survival in Hyperoxia**—A principal finding of this study was that ERK-1/2 was activated in hyperoxia by ATP release, and that this action was critical for survival. ATP-mediated ERK activation was observed within 30 min of exposure to hyperoxia, and its effect was both time- and concentration-dependent.

**Fig. 8.** Effect of PD98059 on survival of HLMVEC in hyperoxia. Cells were preincubated for 30 min with Me2SO or PD98059 (10 μM) and exposed for 24 h or throughout the duration of exposure to hyperoxia (6 days) with inhibitors. Panel A shows phase-contrast images of cells treated: 1) 21% oxygen-exposed cells with Me2SO (0.5 μl per ml of medium, control for PD98059), 2) 21% oxygen-exposed cells with PD98059 (10 μM) for 24 h, 3) 21% oxygen-exposed cells with PD98059 (10 μM) for 6 days, 4) 95% oxygen-exposed cells with Me2SO (0.5 μl per ml media, control for PD98059), 5) 95% oxygen-exposed cells with PD98059 (10 μM) for 24 h and no inhibitor for rest of exposure, 6) 95% oxygen-exposed cells with PD98059 (10 μM) for 6 days. Panel B indicates percent cell death in air or oxygen (6 d), with and without inhibitor. The figure shows a representative experiment performed in triplicate. Results are mean ± S.E. (*) p < 0.05 versus respective untreated hyperoxia control.

**Fig. 9.** Status of ERK phosphorylation by ATP after 24 h of treatment of HLMVEC with PD98059. HLMVEC were treated with PD98059 (10 μM; 24 h) in standard medium. After exposure, cells were treated with ATP (100 μM) for 15 min, lysates prepared, and Western blot performed.

**Fig. 10.** Effect of ATP and hyperoxia on AA release in HLMVEC. HLMVEC were incubated with 0.25 μCi of [3H]arachidonic acid/well in serum-free medium overnight. After washing to remove unincorporated [3H]AA, cells were stimulated with ATP and hyperoxia (30 or 60 min). Total cellular radioactivity and the amount of radioactivity in supernatant were determined by scintillation counting. Results are expressed as percentage of total cellular AA released and are shown as mean ± S.E. (*) p < 0.05 compared with normoxic controls. Data shown represents one experiment (n = 3 per condition) performed three times. *Inset shows inhibition of ATP- and hyperoxia-dependent AA release by suramin (100 μM). Cells were preincubated with suramin for 30 min prior to stimulation. AA release induced by calcium ionophore A23187 (0.5 μg/ml) was the positive control. Additional conditions are as indicated above. Presence of serum is indicated by white columns, whereas absence of serum is indicated by black columns.

**Fig. 11.** Effect of hyperoxic exposure on prostaglandin production and release into extracellular medium. HLMVEC were exposed to hyperoxia (95% O2, sea level pressure) for 2, 24, or 48 h. Following exposure, supernatant medium were collected and analyzed for prostaglandins by ELISA. Results are mean ± S.E. (*) p < 0.05 compared with control n = 6.
for survival. Hyperoxia-induced extracellular signal-regulated kinase (ERK) activation occurs in rat airway epithelial and other cells (52–58). In addition, hyperoxia can cause EGF receptor-dependent ERK activation, leading to enhanced expression of Egr-1, a stress response gene (54). ERK activation by ROS also can occur in bovine aortic endothelial cells and human pulmonary artery endothelial cells (56, 59). Macrophages can be a major source of ROS in hyperoxic lungs (60), and hyperoxia-induced ERK activation can occur in macrophage cell lines (52).

Extracellular ATP can induce proliferation and differentiation in a variety of cells (10, 45), possibly by activation of ERK-1/2 via the dual-specificity kinase mitogen-activated protein kinase/ERK kinase (i.e. MEK). Such activation appears independent of PI3K activity (45). In our system, extracellular ATP also induced ERK-1/2 phosphorylation, and this effect was abolished by the ATP scavengers apyrase and ADA, but not by ADA alone, indicating a specific effect of ATP. Inhibition of pertussis toxin- or cholera toxin-sensitive G-proteins had no effect, suggesting that G1 and G3 classes are not involved. Precedents for toxin-insensitive G-proteins linked to P2Y receptors exist (61, 62). HLMVEC have P2Y receptors on their surface (13), and our results indicate their potential involvement in ATP-dependent ERK-1/2 activation. Various agonists showed activity in the order: ATP $\rightarrow$ ADP $\rightarrow$ ATP $\rightarrow$ UDP $\rightarrow$ ADP $\rightarrow$ ATP $\rightarrow$ UDP, suggesting the involvement of P2Y$_{1}$ or P2Y$_{4}$ receptors. Both ATP and UDP use a common nucleotide receptor, P2Y$_{4}$ (previously known as P2U), to transduce their signals (39). We cannot entirely exclude potential involvement of pathways involving adenosine or AMP, which may indicate that such proteins merely modulate the transport process. By contrast, inhibitors of PI3K, LY294002 and wortmannin, were most effective in decreasing the ATP release by suramin indicated that ATP released by hyperoxia can contribute to enhanced prostaglandin synthesis.

**Mechanism of ATP Release—**Experiments using the chloride channel inhibitor niflumic acid suggested the involvement of ATP-binding cassette transporter proteins like cystic fibrosis transmembrane regulator (CFTR) in hyperoxia-mediated ATP release. However, the relatively small effect of this inhibitor may indicate that such proteins merely modulate the transport process. By contrast, inhibitors of PI3K, LY294002 and wortmannin, were most effective in decreasing the ATP release by hyperoxia. A role for lipid products of PI3K in regulation of ATP release and chloride transport in biliary epithelial cells has been described (71). In the present study, colchicine, a microtubule-disrupting agent, also could inhibit hyperoxia-induced ATP release. These findings indicate that ATP release in HLMVEC likely occurs via a complex mechanism (Fig. 12).

Lung microvascular endothelium is a critical target of hyperoxic injury. In *in vitro* models, this cell frequently shows the most severe damage among the more than 40 cell types present in lung. This report demonstrates direct release of ATP into the...
extracellular space by primary microvascular endothelial cells in hyperoxia. These results further support a critical role for ATP in modulating endothelial cell responses during exposure to altered oxygen concentrations. Extracellular ATP induced activation of ERK, PI3K, and mTOR, primarily through activation of P2Y2 and P2Y12 receptors. Enhanced P2Y1 receptor-mediated glucose uptake, also inhibited by rapamycin, further supported a protective role for mTOR. Moreover, in these cells, ERK activation due to ATP release in hyperoxia also confers a survival advantage.

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