Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth: signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor

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Running title: ATP- and hypoxia-induced fibroblast growth

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*Supported by SCOR # HL 56481 and NIH # HL 14985

ABSTRACT

Important autocrine/paracrine functions for the adenine nucleotides have been proposed in several tissues. We addressed the possibility that extracellular ATP would modulate/mediate hypoxia-induced adventitial fibroblast growth. Acute hypoxia (3% O₂ 10-60 min) increased extracellular ATP concentrations in adventitial fibroblasts and in lung microvascular endothelial cells, and chronic hypoxia (3% O₂, 14-30 days) markedly attenuated the rate of extracellular ATP hydrolysis by ecto-nucleotidase(s). Exogenous ATP stimulated [³H]thymidine incorporation in fibroblasts as did UTP, ADPβ, MeSATP, α,βMeATP and BzATP, indicating that both P2Y and P2X purinoceptors can mediate mitogenic responses. Suramin (100 μM), cibacron blue 3GA (100 μM) and PPADS (100 μM), as well as apyrase (5U/ml) attenuated hypoxia- and ATP-induced and DNA synthesis, indicating activation and a functional role of purinoceptors under hypoxic conditions. ATP-induced DNA synthesis was augmented by hypoxia in an additive fashion, whereas ATP and hypoxia synergistically increased growth factor-induced DNA synthesis, again suggesting that ATP and hypoxia utilize similar signaling pathways to induce proliferation. Indeed, we found, that ATP (100 μM) and hypoxia (3% O₂), induced expression and activation of Egr-1 transcription factor, and both stimuli acted, in part, through a Gαi/ERK1/2-dependent signaling pathway. Suramin, cibacron blue 3GA and apyrase attenuated hypoxia-induced ERK1/2 activation and Egr-1 expression. We conclude that hypoxia induces ATP release from endothelial cells and fibroblasts and that the activation of P2 purinoceptors is involved in the regulation of DNA synthesis by fibroblasts under hypoxic conditions.
INTRODUCTION

Hypoxia has been shown to act as a direct proliferative stimulus for fibroblasts in a variety of organs. This capability of fibroblasts is unusual, at least among mesenchymally derived cells, and appears to be important in normal development, wound healing, fibrosis, as well as in the vascular changes that characterize hypoxic pulmonary hypertension (1-3). With regard to pulmonary artery adventitial fibroblasts, we have shown that among the resident vascular wall cells they exhibit the earliest and most dramatic responses to hypoxic exposure in vivo (4). In tissue culture we have also demonstrated that hypoxia, in the absence of exogenous mitogens, induces proliferation of pulmonary artery fibroblasts, as well as some subpopulations of aortic adventitial fibroblasts. This response is due in large part to Gαi/o mediated activation of a complex network of MAP kinases, whose specific contributions to hypoxia-induced proliferation differ from those of serum-induced growth signals (5). It remains unclear, however, whether either activation or augmentation of the hypoxia-induced growth response is due, at least in part, to autocrine/paracrine responses to factors secreted by fibroblasts during hypoxia, which act through G-protein coupled-pathways.

One factor, which could contribute to such an autocrine loop, is ATP. Purines and pyrimidines (mainly ATP, ADP, adenosine, and UTP) have widespread and specific extracellular signaling actions in the regulation of a variety of functions in many tissues and appear to have key roles in development, proliferation, differentiation, and release of hormones, neurotransmitters and cytokines (6-10). It is also becoming evident that alterations in the physiology of purinergic signaling may result in the development of a variety of pathologies including disorders of the immune system, neurodegenerative and vascular diseases (7). Extracellular ATP can, in fact, stimulate the growth and proliferation of vascular SMC, and this response may play an important role in a variety of vascular diseases (10-12). Importantly, in addition to nerves and circulating blood cells, vascular cells themselves appear to be a potent source of ATP and other adenine nucleotides as these products are known to be released into the extracellular
milleu in response to many vascular stress conditions including ischemia/oxidative stress, flow, and mechanical stretch (13-16). Since vascular cells have been shown to express metabotropic (P2Y) and/or ionotropic (P2X) subtypes of purinergic receptors and since these receptors have been shown to be implicated in mitogenic signaling pathways (8, 9, 17), released ATP could induce cell activation by an autocrine/paracrine mechanism. In fact, the extracellular concentration of ATP and subsequent purinergic activation appears to play a critical role in determining the intracellular signaling setpoint of many key growth-regulating factors (18-20). Further, the concentration of extracellular ATP is rapidly modulated by ecto-nucleotidases, which themselves may be regulated by environmental factors. Therefore, the release of ATP and modulation of its degradation by environmental or chemical stimuli may contribute to the setpoint of cellular signaling pathways regulated by P2 receptors and raises the possibility that extracellular ATP is a critical modulator of signal transduction pathways operating to control proliferative responses. It remains unclear, however, whether hypoxia actually increases extracellular concentrations of ATP in fibroblasts and whether ATP is a component of the hypoxia-induced proliferative response.

It is known that hypoxia-induced cell proliferation is dependent on expression of specific transcription factors. However, the transcription factors involved in regulating the proliferative response of non-transformed vascular fibroblasts to moderate levels of hypoxia have not been elucidated. It is remarkable, that many stress conditions which can trigger cell proliferation, including hypoxia, mechanical stress and inflammation, can induce early growth response Egr-1 transcription factor (21-23) which has been shown to be critical for proliferation and migration in many cell types (22, 24, 25). These stress conditions have also been shown in independent experiments to induce ATP release in a variety of cell types (7, 13, 26-28). Thus, it seems possible that hypoxia itself and/or hypoxia-induced increases in extracellular ATP could act to increase Egr-1 expression and activity, which might be necessary for DNA synthesis. However, little information exists as to whether ATP or hypoxia directly regulates Egr-1 expression in adventitial fibroblasts.

The purpose of this study was to test the hypothesis that hypoxic stress would increase extracellular ATP concentration leading to the creation of a “purinergic network” where local ATP
release, degradation, and stimulation of purinergic receptors operate to control fibroblast proliferation under hypoxic conditions. In addition, we hypothesized that ATP and hypoxia would up-regulate expression and activation of the Egr-1 transcription factor through Gαi-initiated, ERK1/2-dependent pathways. We used primary cultures of pulmonary artery adventitial fibroblasts to examine the effects of hypoxia and ATP on growth responses, Egr-1 expression, and on its DNA binding activity. We evaluated the effects of hypoxia on extracellular ATP concentration and on the enzymes (ecto-ATPase(s)) that catalyse extracellular ATP degradation. Finally, the role of ATP in hypoxia-induced growth, Egr-1 expression and ERK1/2 phosphorylation was determined using pharmacological inhibitors. Our findings strongly support the possibility that extracellular ATP plays an important role in modulating hypoxic proliferative responses and that it significantly modulates the response of the fibroblast to other mitogens that may be present in the hypoxic environment.
EXPERIMENTAL PROCEDURES

Cell culture

Pulmonary artery adventitial fibroblasts were isolated from tissue explants of 120- to 180-day gestational bovine fetuses as previously described (29). Briefly, adventitia of lobar pulmonary artery was mechanically separated from the media and capillaries, and chopped into small pieces (1mm²) which were then placed in 6-well plastic culture dishes (one per well). When numerous cells were observed around explants, tissue was removed, and cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 20 mM L-glutamine (Cellgro, VA), non-essential amino acids, (1:100, v/v), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical; St.Louis, MO) and 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA). Cell cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C, and medium was changed every three days. For expansion, fibroblasts were cultured to confluence, harvested with trypsin-EDTA solution (0.2g/l-0.5g/l), and replaced at 1:4 ratio. All studies were performed on cells between passages 2-6. For Northern and Western blot analysis, cells were plated at a density of 5x10⁵ cells/cm², cultured to 80% of confluence and then growth arrested in 0.1% FBS/DMEM for 72 hrs, and then subjected to the experimental conditions described below.

Human lung microvascular endothelial cells (HLMVEC) were purchased as frozen primary cultures from Clonetics Ltd (Walkersville, MD). Cells were cultured in endothelial cell basal medium (EBM-2) supplemented with VEGF, hFGF, EGF, hydrocortisone, ascorbic acid, IGF, GA1000 and fetal bovine serum as per manufacturers protocol. Hypoxic and normoxic bovine pulmonary artery endothelial cells were generous gift from Dr. H.Farber (Boston University).

DNA synthesis analysis

DNA synthesis was determined by [methyl-³H]thymidine incorporation. Cells were plated in 24 well plates at a density of 15 x 10⁴ cells /well in DMEM, supplemented with 10% FBS. In 24 hrs cells were
rinsed with phosphate-buffered saline (PBS), and incubated in serum-deprived (0.1% FBS) DMEM for 72 hrs. Then cells were stimulated with ATP (10^{-9}-10^{-3} M) under either normoxic (21% O\textsubscript{2}, 5% CO\textsubscript{2} balance N\textsubscript{2}) or hypoxic (1%, 3% or 10% O\textsubscript{2}, 5% CO\textsubscript{2} balance N\textsubscript{2}) conditions at 37°C in Plexiglas chambers (Bellco Glass, Vineland, NJ) in the presence of 1.0 μCi of [methyl-\textsuperscript{3}H] thymidine (1 mCi/ml, 20Ci/mmol, NEN Life Science Products, Boston, MA) for 24 hrs. The effects of UTP, ADP\textbeta\textsubscript{5}, Me\textalpha\textbeta\textsuperscript{m}ATP and BzATP (Sigma Chemical) on DNA synthesis were tested under normoxic conditions in a similar manner. When the effect of P2 receptor antagonists were tested, suramin, cibacron blue 3GA or PPADS (Sigma Chemical) were added at the concentrations of 100 μM 30 or 60 min before stimulation with ATP and/or hypoxia. For the experiments with apyrase cells were preincubated for 2 hrs with a mixture of apyrases grade VI and VII (Sigma Chemical) at a final concentration of 5U/ml media. In the experiments aimed to prevent ATP hydrolysis ARL67156 (300 μM) (30), or ATP-regeneration system (0.1 mg/ml creatine kinase, 4 mM phosphocreatine and 0.025 mg/ml myokinase (31)) were added to the incubation media. At the end of incubation cells were washed twice with PBS, incubated with 0.5 ml of 0.2M perchloric acid for 3 min, washed with 1 ml PBS, and lysed in 0.3 ml of 1% SDS/0.1M NaOH. Samples were harvested, mixed with liquid scintillation cocktail (Ecoscint H, National Diagnostics, Atlanta, GA), and incorporated radioactivity was counted (cpm/min) in a liquid scintillation counter (Beckman LS 6500).

**Northern Blot Analysis**

Total cellular RNA was extracted with either “TriReagent” solution (Sigma Chemical) or with RNeasy kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer’s instructions. RNA pellets were dissolved in DEPC-treated water and heated for 10 min at 55°C. RNA concentration was determined by measurement of absorbency at 260 and 280 nm. Northern blot analysis was carried out by using NorthernMax-Gly\textsuperscript{TM} blotting kit (Ambion, Austin, TX). 10-15 μg of total RNA were treated with glyoxal loading buffer and separated by 1% agarose formaldehyde gel electrophoresis at 85 V constant voltage.
Following electrophoresis, RNA was transferred to Hybond™– N+ membrane (Amersham, Pharmacia Biotech) by capillary transfer for 2-2.5 hrs. After transfer, membrane filters were briefly rinsed in a transfer buffer and UV cross-linked (UV Stratalinker, Stratagene, La Jolla, CA). Membranes were prehybridized in hybridization solution (UltraHyb, Ambion), for 45 min at 68°C and hybridized with 50-100 ng/ml of digoxygenin-UTP-labeled RNA overnight at 68°C. After washing, hybridized filters were probed with anti-digoxygenin antibodies (F(ab’)2 fragments) conjugated to alkaline phosphatase (1:1000 dilution, Roche Molecular Biochemicals, Indianapolis, IN). Bands, corresponding to Egr-1 mRNA were detected using Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5’-chloro)tricyclo[3.3.1.13.7]decan}-4-yl phenyl phosphate (CSPD) chemiluminescent substrate (Roche Molecular Biochemicals). The antisense RNA probe for bovine Egr-1 was produced by in vitro transcription using digoxygenin-labeling kit (Roche Molecular Biochemicals). 1.4-kilobase fragment of Egr-1 cDNA, ligated into pBluecript (+) vector was obtained from American Type Tissue collection (Rockville, MD). cDNA template was prepared by linearization of the plasmid by KpnI digestion upstream of T7 promoter, and digoxygenin-UTP-RNA probe was synthesized by reverse polymerase reaction. To provide normalization for RNA loading and transfer, after UV cross-linking blots were stained with 0.1% methylene blue/0.5% acetic acid, and bands, corresponding to 18S rRNA were scanned. Alternatively, blots were double-probed with Egr-1 and β-actin digoxygenin-labeled RNA probes and normalized per β-actin mRNA.

**Preparation of Nuclear Extracts**

Fibroblasts were grown and serum starved as described above. Culture media was then replaced with fresh serum-free DMEM and cells were stimulated with either ATP (100 μM) or hypoxia (3% O2) for 2, 4, 6, 12 and 24 hrs. At the end of incubation period, cells were washed twice with ice-cold PBS, scraped off the dishes and pelleted by centrifugation at 500 x g for 3 min at 4°C. Nuclear extracts were prepared using NE-PER™ extraction reagents (Piers, Rockford, IL) according to the manufacturer’s recommendations. Nuclear extracts and cytosolic fraction were snap-frozen in liquid nitrogen in aliquots.
and stored at −80°C. Protein concentration was determined by Bradford method using Bio-Rad protein assay kit (Bio-Rad Life Science Product) with bovine serum albumin as a standard.

**Western blot analysis of nuclear extracts**

15 μg of nuclear protein extract was separated by 8% SDS-polyacrylamide gel electrophoresis, and electroblotted to the PVDF membrane (Hybond P, Amersham Pharmacia Biotech) in Tris-glycine buffer (25 mM Tris-base, 0.2M glycine, 25% (v/v) methanol, pH 8.3) at 350-400V, for 4 hrs at 4°C. Following transfer, membrane filters were stained with Ponceau S (Sigma Chemical) to verify equal protein loading and blocked with 5% nonfat dry milk in TBS-Tween (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05%Tween 20) for 1 h at room temperature. Membrane filters were probed with Egr-1 rabbit polyclonal antibodies (C-19, Santa Cruz Biotechnology, CA) at a dilution of 1:1000 in TBS-Tween with 5% milk for 1 h at 4°C. After intensive washing in TBS-Tween, membrane filters were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:20,000 dilution, Amersham Pharmacia Biotech), and immunoreactive bands were detected by Renaissance ECL detection kit (NEN Life Science Products) followed by exposure to Hyperfilm (Amersham Pharmacia Biotech).

**Evaluation of phospho-ERK 1/2**

Cells were plated in 6-well cell culture plates at a density of 100 × 10^3 cells/well, cultured to near confluence and then were serum-starved in 0.1% FBS/DMEM for 72-96 hrs. To evaluate the involvement of Gi proteins in ERK1/2 activation, cells were treated with pertussis toxin (PTx, 100 ng/ml) for 20-24h. Cells were incubated under either normoxic (21% O₂) or hypoxic (3% O₂) conditions in fresh serum-free DMEM in the presence or absence of ATP (100 μM) for 10 min. After incubation, cells were washed twice with ice-cold PBS and lysed with cold Tris-HCl buffer (40 mM pH 7.5), containing 0.25 M sucrose, 3 mM EGTA, 3 mM EDTA, 50 μM β-mercaptoethanol, 1 mM PMSF and complete protease inhibitors cocktail (Calbiochem, La Jolla, CA). Cell lysates were centrifuged at 7500 × g for 10 min at 4°C, and
supernatant fractions were collected and stored at –80°C. Protein concentration was determined by using Bio-Rad protein assay kit with bovine serum albumin as a standard. Samples of total cell protein (5μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membrane and probed with rabbit polyclonal antibodies against phospho-ERK1/2 (Tyr202/Thr204), 1:1000 dilution, (New England Biolabs, Beverly, MA) overnight at 4°C. Blots were then washed with TBS-Tween buffer and incubated with mouse anti-rabbit peroxidase-conjugated IgG 1:10,000 dilution, (Amersham), for 1 h at room temperature. Immunoreactive bands were detected by ECL detection kit (Renaissance, NEN Life Science Product) followed by exposure to Hyperfilm For detection of non-phosphorylated form of ERK, membranes were stripped of the bound antibodies with buffer containing 62.5 mM Tris-HCl, pH 6.8; 2% SDS and 100 μM β-mercaptoethanol, blocked with 5% nonfat dry milk in TBS-Tween and reprobed with rabbit polyclonal antibodies against total ERK1/2 (1:1000), (New England Biolabs).

Electrophoretic Mobility Shift Assay
To detect DNA-protein complexes, nuclear extracts were incubated with 32P-labelled double-stranded oligonucleotide probe, containing two copies of consensus (5’-GGA TCC AGC TAG GGC GAG CGG TAG CGA-3’) or mutant ((5’-GGA TCC AGC TAG GGC GAG CGG TAG CGA-3’) of Egr-1 binding motifs (Santa Cruz Biotechnology, CA). Oligonucleotides were 5’-end labeled by using [γ-32P]-ATP (3000Ci/mmol, 10 mCi/ml, NEN Life Science Product) with T4 polynucleotide kinase according to the manufacturer’s instructions (New England Biolabs). Labeled oligonucleotides were purified using Chroma Spin columns-10 (Clontech, Palo Alto, CA). The binding reaction was carried out for 30 min at room temperature in a 20 μl of mixture containing 10 mM Tris (pH 8.0), 5% glycerol, 50 mM NaCl, 5 mM MgCl2, 10 μM ZnCl2, 2 mM DTT, 0.1 mM EDTA, 1 μg of poly (dI-dC) (Roche Molecular Biochemicals), 0.5-1 ng of the 32P-labeled DNA (8000 cp) and 5 μg of nuclear protein. For the competition study, incubation mixtures containing 80-fold excess of unlabeled Egr-1 oligonucleotides...
were used. To test specificity of Egr-1 DNA binding complexes antibody blocking experiments were carried out. Nuclear extracts were preincubated with 2 μg of specific anti-Egr-1 polyclonal IgG (C-19X, Santa Cruz Biotechnology) for 45 min at room temperature, and then subjected to the binding reaction. Protein-DNA binding complexes were separated from free DNA probe by native 4% polyacrylamide gel electrophoresis in 0.5×TBE running buffer (0.045 M Tris-borate, 0.001 M EDTA (pH 8.5)), at 30 mA constant current for 3-3.5 hrs. Gels were vacuum-dried and exposed to Hyperfilm (Amersham Pharmacia Biotech) in a cassette with two intensifying screens at –80 °C for 6-20 h.

**Determination of extracellular ATP**

Total ATP content in the extracellular media was detected with luciferase-luciferin kit (Analytical Luminescence Laboratory, Sparks, MD) using a Monolight 3010 luminometer (Analytical Luminescence Laboratory). Cells in 60 cm² Petri dishes (at a density of 500 × 10³ cells/cm²) were exposed to either normoxic (21% O₂) or hypoxic (3% O₂ and/or 1% O₂) conditions for the periods of time indicated in Figure 1. At the beginning of the experiment, media was drained from the cells and fresh media, which had been pre-equilibrated with the appropriate gas mixture, was infused onto the cell monolayer. Following incubation, 1 ml of conditioned media was collected into chilled polypropylene tubes (Sigma Chemical), and centrifuged at 12,000 × g for 10 min to remove any cell debris. Individual 100 μl aliquots were taken, heated at 95 °C for 1 min, and the luciferin-luciferase assay was carried out. The sampled luminescence was compared with an ATP standard curve performed in each individual experiment.

**Determination of the products of extracellular ATP hydrolysis**

Cells were cultured under normoxic (21% O₂,) or hypoxic (3% O₂) conditions for 3-4 passages and then were plated in 12-well culture dishes (40 × 10³ cells/well). Confluent cells were growth arrested for 72 hrs like described above and washed twice with 1 ml of PBS (37°C). To determine products of [α-³²P]-ATP hydrolysis (specifically, [α-³²P]-ADP, [α-³²P]-AMP and ³²Pi) culture medium was replaced with 0.6 ml
of serum-free DMEM (25° C) and the reaction was initiated by adding 0.1 mM ATP and 5 μCi of [α-\(^{32}\)P]-ATP (30 Ci/mmol, 2 mCi/ml, NEN Life Science Products). After indicated periods of time (Fig. 2) two μl aliquots of culture medium were taken and spotted onto polyethylenamine cellulose-coated TLC plates (Sigma Chemical) and chromatographed in 1M LiCl for 2.5-3 hrs. Radioactive spots corresponding to adenine nucleotides and Pi were detected by autoradiography using Hyperfilm (Amersham) and were verified to be at the same position as those of unlabeled adenine nucleotide standards.

Data analysis

Density of bands of Western and Northern blots film images was determined by using NIH Image 1.58 program. Data are expressed as average mean ± standard error (S.E.); n equals the number of replicates in one experiment or a number of observations in independent experiments. To evaluate significance of the obtained data, analysis of variance between groups of data was performed by Student–Newman-Keuls test followed by one-way ANOVA.
RESULTS

Hypoxia Increases Extracellular ATP Concentration

We first sought to determine if hypoxia would stimulate ATP release from fibroblasts (Fig.1). Quiescent growth arrested adventitial fibroblasts were exposed to pre-equilibrated normoxic or hypoxic media (21, 3, and 1% O2) for up to 24 hrs. Zero minute data represent the concentration of ATP in the pre-equilibrated media before it was added to cells. We found that ATP accumulated in the media of unstimulated fibroblasts even under normoxic conditions (note 24 hrs concentration), consistent with constitutive release of ATP, as has been observed in human skin fibroblasts and another cell types (32, 33). Exposure of fibroblasts to hypoxic conditions, which stimulated cell growth (3 and 1% O2) resulted in increased accumulation of ATP in the media, with the highest concentration seen at 10 minutes and with persistently increased concentrations observed even at 24 hrs (Fig.1A).

Other cells in the vessel wall also may be a source of ATP for adventitial fibroblasts. Since increased extracellular ATP concentrations have been observed in endothelial cells exposed to hemodynamic stress, and since, in small vessels, they can be the source of many paracrine factors which influence fibroblast function (28), we evaluated the effect of hypoxic exposure on endothelial extracellular ATP levels. We found that hypoxia caused increases in the concentration of ATP in the media of endothelial cells which were about 10 fold higher than in fibroblasts (Fig.1B). The time course of accumulation was slightly different than that observed in fibroblasts, with a peak at 30 min and no differences in extracellular ATP concentration between normoxic and hypoxic cells at 24 hrs.

Chronic hypoxia decreases ecto-nucleotidase(s) activity

Extracellular ATP concentrations are known to be tightly controlled by ecto-nucleotidase(s) which rapidly degrade purine and pyrimidine nucleotides in the extracellular space. Thus, the concentrations of ATP
available for stimulation of purinergic receptors could be regulated by changes in the degradation of extracellular ATP. We considered the possibility that hypoxia would decrease ecto-ATPase(s) activity in either fibroblasts or endothelial cells, thus slowing the rate of extracellular ATP degradation. Utilizing a technique, which allowed evaluation of products of ATP hydrolysis, we evaluated the effects of acute and chronic hypoxic exposure on ATP degradation in cultured cells. We found that brief hypoxic exposure (<24 hrs) had no effect on ecto-ATPase activity in either fibroblasts or endothelial cells (data not shown). However, long-term hypoxic exposure (3% for ≥14 days), as might occur in various pathophysiologic states including hypoxia-induced vascular remodeling, significantly decreased ecto-ATPase(s) activity in both fibroblasts and endothelial cells. As shown in Fig. 2A, addition of [α-32P]-ATP to adventitial fibroblasts maintained under normoxic conditions resulted in rapid hydrolysis of [α-32P]-ATP to [α-32P]-ADP, [α-32P]-AMP, and 32P with $t_{1/2}(\text{ATP}) = 10.6 \pm 1.8 \text{ min}$ and $V_o=25.6\pm2.10 \text{ nmol/min/10}^6 \text{ cells}$ (Panel A, line 1 and 4). In cells exposed to chronic hypoxia, a delayed rate of ATP hydrolysis was observed ($t_{1/2}(\text{ATP}) = 17.6 \pm 2.8 \text{ min}$ and $V_o=19.8\pm1.95 \text{ nmol/min/10}^6 \text{ cells}$ in chronically hypoxic cells under hypoxic conditions (Panel A, lines 2 and 5), $t_{1/2} \text{ (ATP)} = 22.0 \pm 2.2 \text{ min}$ and $V_o=16.0\pm1.82 \text{ nmol/min/10}^6 \text{ cells}$ in chronically hypoxic cells under normoxic conditions (Panel A, lines 3 and 6)). A similar effect of chronic hypoxia was observed in endothelial cells (normoxia $t_{1/2}(\text{ATP}) = 1.75 \text{ min} \pm 0.25 \text{ min}; V_o=77.45\pm6.35 \text{ nmol/min/10}^6 \text{ cells}$ versus hypoxia $t_{1/2}(\text{ATP}) =7.2 \pm 1.6 \text{ min}; V_o=35.69\pm2.93 \text{ nmol/min/10}^6 \text{ cells}$ (Fig. 2B).

**Extracellular ATP Induces DNA Synthesis in Adventitial Fibroblasts through both P2Y and P2X receptors; Hypoxia Augments the Response**

Although extracellular ATP has been reported to stimulate proliferation in a wide-range of cell types including endothelial cells, vascular smooth muscle cells (SMC), and fibroblast cell lines, its effect on vascular adventitial fibroblast proliferation, either alone or in the presence of hypoxia, has not been examined. We found that ATP stimulated increases in thymidine incorporation at doses between $10^{-6}$ and $10^{-3}\text{M}$ in quiescent, serum-starved, adventitial fibroblasts under normoxic conditions (21%O₂) (Fig. 3A).
Under hypoxic conditions (3% O₂), thymidine incorporation was increased even in the absence of ATP, consistent with our previous observations (5). We found that hypoxia augmented the proliferative effects of ATP such that there was a shift in the dose response curve to the left by about one order of magnitude (Fig. 3A). The effect of ATP on proliferation in the presence of the ecto-ATPase inhibitor ARL67156 and an ATP-regenerating system (creatine kinase, phosphocreatine and myokinase) was also evaluated to more carefully delineate the concentrations at which ATP stimulates [³H]thymidine incorporation since considerable degradation of ATP likely takes place over the 24 hrs time period required for the assessment of DNA synthesis. In the presence of ARL67156, the concentration-dependent curve was shifted significantly to the left, and demonstrated that DNA synthesis is initiated at concentrations as low as 10⁻⁷⁻¹⁰⁻⁶ M. A similar shift in the concentration-dependent curve was also observed in the presence of ATP-regenerating system (Fig. 3B).

Because the concentration range of ATP found to stimulate DNA synthesis could activate both P2Y and P2X receptors (11, 34, 35), we evaluated the effects of selective P2Y and P2X receptor agonists on DNA synthesis. We found that the P2Y receptor agonists UTP, ADPβS, and the P2Y/P2X agonist MeSATP stimulated increases in thymidine incorporation which were nearly equivalent to those observed with ATP at doses causing a maximal effect (Fig. 3C). In addition the P2X7 selective agonist BzATP caused a less, but still significant increase in DNA synthesis. The P2X1,3 receptor agonist α,βmeATP, stimulated DNA synthesis only when used at 10⁻³M. Adenosine, on the other hand, had no significant effect on DNA synthesis suggesting that P2Y and P2X purinoceptors, but not P1 purinoceptors, were likely involved in ATP-stimulated adventitial fibroblast proliferation.

In contrast to the effects exerted by hypoxia and ATP (Fig. 3A), we observed synergistic interactions between ATP and purified peptide mitogens and between hypoxia and purified peptide mitogens (Fig. 3D).
Since extracellular ATP concentrations were increased in response to hypoxia and an autocrine/paracrine role of ATP has been demonstrated in another cell types, we tested the possibility that ATP may mediate, at least in part the proliferative effect of hypoxia. We evaluated the effect of P2 receptor antagonists as well as apyrase on hypoxia–induced DNA synthesis. We found that preincubation with the P2 receptor antagonists suramin (100 μM, 30 min), cibacron blue 3GA (100 μM, 30 min) or PPADS (100 μM, 60 min) attenuated [3H]-thymidine incorporation in response to ATP, hypoxia (3% O₂), and in response to combined stimulation of cells with ATP and hypoxia (Fig. 4A). The inhibition of DNA synthesis was not due to cytotoxic effects of suramin, cibacron blue 3GA or PPADS, because no change in lactate dehydrogenase levels, or the ability to exclude trypan blue, was observed (data not shown). In addition we found that apyrase, which specifically hydrolyses ATP and ADP in the incubation media, reduced [3H]-thymidine incorporation in response to ATP and hypoxia by ~50%, again suggesting that extracellular ATP contributes to hypoxia-induced DNA synthesis in adventitial fibroblasts (Fig. 4B).

**ATP and Hypoxia Induce Egr-1 mRNA Expression in Adventitial Fibroblasts**

Egr-1 is a transcription factor that is commonly induced under stress conditions and that has been demonstrated to be critical in stress-induced proliferative responses. Because hypoxia increased extracellular ATP, we tested the possibility that Egr-1 would be a downstream signaling mediator induced in response to hypoxia and ATP in adventitial fibroblasts. Using Northern blot analyses, we found that both ATP (10 μM) and hypoxia (3% O₂) markedly induced Egr-1 mRNA and that the ATP-mediated response was augmented under hypoxic conditions (Fig. 5). We also found that the pyrimidine nucleotide UTP, whose extracellular concentration can be elevated in response to acute vascular stress, induced Egr-1 mRNA with similar efficiency to ATP. Factors including PDGF, FBS and PMA, all previously shown to stimulate Egr-1, were used as positive controls and for qualitative comparison purposes.
Next, we compared time- and dose-dependent profiles for both ATP and hypoxia on Egr-1 mRNA expression. ATP was shown to stimulate Egr-1 mRNA in a time- and dose-dependent fashion. A maximal induction of Egr-1 mRNA levels of approximately 8.5-fold over basal was observed at 100 μM ATP (Fig. 6, Panel A). At this concentration Egr-1 mRNA was induced within 15 min, peaked at 1 h, and returned to basal level by 4 hrs. Hypoxic exposure alone also stimulated an increase in Egr-1 mRNA expression in quiescent serum-starved adventitial fibroblasts. A significant increase was seen at O₂ concentrations of 10% with a maximal effect observed at 1% (Fig. 6, Panel B). Using both 1% and 3% oxygen concentrations, we found the time course and magnitude of activation by hypoxia to be remarkably similar to that of ATP with a 7-fold induction observed within 30 min and a decline to baseline by 6 hrs.

**P2 Purinergic Receptors, Gαi Proteins and ERK 1/2 Mediate Both ATP- and Hypoxia-Induced Egr-1 mRNA Expression**

Since we have previously shown Gαi and ERK1/2 to be important in hypoxia-induced proliferation, and because P2 receptor antagonists attenuated hypoxia-induced proliferation (above), we studied the effects of pertussis toxin (PTx), known to selectively inhibit Gαi/o-mediated signaling, a specific MEK-1 inhibitor PD98059, P2 receptor antagonists (suramin, cibacron blue, PPADS) and apyrase on hypoxia- and ATP-induced Egr-1 mRNA levels. Northern blot analysis demonstrated that preincubation with Pertussis toxin (100 ng/ml, 20-24 hr) attenuated ATP-and hypoxia-induced Egr-1 mRNA expression by 40 and 48% respectively (Fig.7) indicating that activation of Gαi/o, as well as PTx-insensitive Gαi/o proteins may underlay ATP- and hypoxia-induced Egr-1 expression (Panel A). The MEK-1 inhibitor PD98059 (10 μM, 1 h) attenuated ATP and hypoxia-induced responses by 63 and 58% respectively. Preincubation with suramin (Panel A), cibacron blue 3GA (data not shown) and apyrase (Panel C) inhibited both ATP- and hypoxia-induced Egr-1 expression. PPADS, which is thought to act preferently on P2X receptors, and only on some subtypes of P2Y receptors, was less effective in blocking hypoxia-
induced Egr-1 expression (Panel B). Collectively, these data suggest that P2 receptor activation by the release of endogenous ATP may occur under hypoxic conditions.

To further examine the possibility that both ATP and hypoxia utilize Gαi-initiated ERK1/2 activation to control proliferation and Egr-1 expression, we evaluated the effects of pertussis toxin on ATP- and hypoxia-induced ERK1/2 phosphorylation (Fig. 8 A, B). As expected, we found that both ATP and hypoxia induced ERK1/2 activation and the combination of exogenous ATP and hypoxia enhanced ERK1/2 phosphorylation in an additive manner. Pertussis toxin partially inhibited the hypoxia-induced response as well as the ATP-induced response under normoxic and under hypoxic conditions. In addition we found that suramin and cibacron blue 3GA significantly attenuated ERK1/2 phosphorylation in response to ATP and hypoxia, but PPADS had less effect on ATP-induced ERK phosphorylation (Fig. 8C). Finally, we found that apyrase almost completely attenuated ATP-induced ERK1/2 phosphorylation and attenuated the hypoxic response at least by 30% (Fig. 8 E, D). Collectively these findings suggest that ATP and hypoxia activate Egr-1 expression and proliferation through both Gαi-dependent and -independent activation of ERK1/2, and that ATP release and purinoceptor activation contribute to hypoxia-initiated responses.

**Hypoxia and ATP Increase Egr-1 Protein Level and DNA Binding Activity**

To determine if stimulation of fibroblasts with ATP or hypoxia resulted in increased Egr-1 protein levels, Western blot analysis of nuclear extracts isolated from control cells as well as cells exposed to either ATP or hypoxia for 0, 2, 4, 6 and 24 hrs were performed (Fig.9, Panel A). Immunoblotting of nuclear fractions with Egr-1 antibodies revealed protein bands with an apparent molecular weight of 82 kDa, which corresponds to Egr-1. In the cytosolic fraction only trace amounts of Egr-1 protein were detected (data not shown). Responses varied over time with hypoxia causing a more prolonged increase in Egr-1 protein levels than ATP. In nuclear extracts obtained from ATP- and hypoxia-treated cells, an additional protein
band appeared after 2 hrs of stimulation, which became more visible at 4 and 6 hrs suggesting that along with increased protein expression, posttranslational modification(s) of Egr-1 may occur.

To demonstrate that increased Egr-1 protein expression in the nucleus was associated with a specific interaction with its DNA recognition site, we performed electrophoretic mobility supershift assays (EMSA). Based on the immunoblotting data (Fig. 9, Panel A), we chose the stimulation condition that had resulted in the highest Egr-1 protein levels (100 µM ATP for 2 hrs and hypoxia (3% O₂) for 4 hrs). Again, nuclear extracts obtained from PMA-treated cells (10 nM, 4 hrs) were used to provide positive controls for the assay conditions. EMSA of nuclear extracts isolated from ATP- and hypoxia-stimulated cells revealed 3 visible DNA-protein complexes (Fig. 9, Panel B, “-” lines). Pre-incubation of nuclear extracts with Egr-1 antibodies (Fig. 9 Panel B, “+” lines) demonstrated the capability of protein from the upper band to form complexes with reduced electrophoretic mobility indicating that protein in these DNA binding complexes corresponds to Egr-1. To further evaluate the specificity of DNA-protein interaction, we carried out binding reactions in the presence of 80-fold excess unlabeled oligonucleotides, containing a consensus Egr-1 binding sequence. We found that radioactivity in the Egr-1 band as well as in one of the lower bands was markedly attenuated (Fig.9, Panel C, Egr-1 bound and asterisk (*)). Since the protein comprising one of the lower bands is observed in equal amounts in control and in stimulated cells, it is possibly a constitutively expressed nuclear factor enabling and/or specifically interacting with Egr-1 DNA binding sites. Finally, we demonstrated that ³²P-labelled oligonucleotides containing mutant Egr-1 binding sequence was ineffective in interacting with either Egr-1 or with a constitutively expressed nuclear protein suggesting that the lower band (Fig. 9, Panel D, two asterisks, (**) ) represents non-specific DNA binding complexes.
DISCUSSION

Activation and proliferation of fibroblasts under conditions of reduced oxygen tension in the local environment is thought to play a critical role in a variety of fibrotic conditions (1-4). However, it remains unclear as to how hypoxia exerts its effects within the local environment and/or intracellularly to effect changes in proliferation. Previous investigations demonstrated that G-protein activation was a critical upstream event in hypoxia-induced proliferation and that subsequent signaling through MAPK, PI3-kinase and PKC pathways was involved in the proliferative response. Other studies demonstrated that extracellular ATP, acting through G protein-coupled receptors (GPCR), could potentially modulate MAPK signaling and proliferation in SMC (7, 10, 36-38). The present study was therefore undertaken to address the hypothesis that hypoxia would stimulate activation of a local purinergic signaling network whereby increases in cellular ATP release, impaired degradation of released ATP, and stimulation of P2 purinoceptors would operate to either control or modulate fibroblast proliferation under hypoxic conditions (Fig. 10). Our data demonstrate that indeed hypoxia can increase extracellular ATP through enhanced release and impaired degradation (Fig. 1, 2). Further, ATP exerts effects on cell signaling and proliferation which are similar to those effected by hypoxia, and the effects of hypoxia and ATP can be abrogated by reducing extracellular ATP and/or blocking G-protein-coupled signaling (Fig. 4). Thus, the findings of the present study suggest that ATP is a released by fibroblasts (and by endothelial cells) under hypoxic conditions and that activation of P2 purinoceptors is involved in the regulation of DNA synthesis under hypoxic conditions.

Investigations in other cell systems have also demonstrated that extracellular nucleotides can function as autocrine-paracrine mediators of a number of cell responses, and that their appearance in the extracellular microenvironment can result from cell lysis, exocytosis of nucleotide-containing granules, or efflux
through membrane transport proteins (7, 36). Recent studies indicate that relatively large amounts of ATP, as well as UTP, are released by mechanical stimulation (e.g. shear stress, hypotonic swelling, or stretch) of epithelial cells, endothelial cells, smooth muscle cells, glial cells, fibroblasts and hepatocytes, and that the presence of these nucleotides in the extracellular medium promotes activation of P2 purinoceptors (13, 15, 27, 38-43). Our data demonstrate that hypoxia induces release of ATP into the extracellular milieu from both adventitial fibroblasts and endothelial cells, and that the increase in ATP concentrations is not the result of cell lysis. Though the measured ATP concentrations in the conditioned media are similar to those previously reported in other stimulated cell systems, they seem to be lower than the threshold for purinoceptor activation noted in this and other studies (32). However, as has been mentioned by many others, and elegantly shown in recent studies, local ATP concentrations near the cell surface and accordingly near the purinoceptors are much higher than those measured in the conditioned media (44). In addition, our data suggest a constitutive basal release of ATP by fibroblasts into the extracellular space, as has been recently demonstrated for the other cell types, which could act to modulate responses to other local stimuli even in the absence of hypoxia (16, 18, 32). Thus, our data indicate that despite ongoing ATP hydrolysis by ecto-nucleotidases, acute hypoxia induces significant increases in extracellular ATP, supporting the possibility of autocrine-induced changes in purinoceptor activation and subsequent cell signaling. In addition and in a broader physiologic context, they also support the possibility that other sources of ATP, such as might be derived from the endothelial cells or from the sympathetic nerves in a vascular adventitia (9, 11, 27, 45) could also contribute to high local concentrations of ATP and thus cooperate in adventitial fibroblast activation and proliferation under hypoxic conditions.

An intriguing question regarding ATP release in response to hypoxia is whether ATP transport through the plasma membrane in vascular cells is coupled to the mechanism of oxygen sensing. An essential role for ATP acting as a sensory mediator has been proposed for several cell types. It has been demonstrated that in the rat carotid body, co-release of ATP and acetylcholine (ACh) underlies the mechanism of
chemoreception (46). Autocrine ATP release is involved in shear stress-induced JNK stimulation in SMC (14), in hydrostatic control of rabbit urinary bladder tone (47), in mechanical stress mediated calcium mobilization in polarized airway epithelial cells (15) and in cell volume regulation (48). Thus, these observations together with our data raise the possibility that extracellular nucleotides play an important role in transducing or modulating a variety of hypoxia-induced responses in vascular cells.

The metabolism of extracellular ATP by ecto-nucleotidase(s)/ecto-ATPases plays an important role in the regulation of purinergic signaling. Recent observations of Moser et al. provide evidence that the antiproliferative action of angiostatin is related to the inhibition of endothelial cell surface, $F_1$-$F_0$ ATP synthase (49). In addition, it has also been demonstrated that certain pathophysiologic conditions, especially those associated with endothelial cell activation, can actually decrease ATP-diphosphohydrolase (ATPDase) activity (50). In support of the possibility that hypoxia could effect this enzyme system are observations that ATPDase activity in COS-7 cells is decreased following exposure to $H_2O_2$ and oxygen radicals (51). However, the effects of acute or chronic hypoxia on ecto-nucleotidase activity have not been previously reported. Our data provide new evidence that chronic hypoxic exposure of pulmonary artery adventitial fibroblasts and endothelial cells can decrease the rate of extracellular ATP hydrolysis, suggesting that regulation of ecto-nucleotidase activity by hypoxia could be an additional mechanism that contributes to the concentration of various extracellular nucleotides levels in the vascular wall. Delayed ATP hydrolysis under hypoxic conditions could cause changes in the ratio between extracellular concentrations of purine nucleotides. Since these different phosphorylation products (ATP, ADP, AMP and adenosine) activate different purinoceptor subtypes, our observations suggest that hypoxia-induced dysregulation of nucleotidases could lead to diverse changes in intracellular signaling and responses. Other extracellular enzymes such as ecto-nucleoside diphosphokinases also can contribute to metabolism of extracellular nucleotides, and future studies need to evaluate the effects of hypoxia on these enzymes.
Little is known of the receptors through which ATP stimulates proliferation in fibroblasts (33). In vascular SMC ATP-induced proliferation is thought to be mediated largely through P2Y receptors acting through ERK1/2 and phosphatidylinositol 3-kinase signaling pathways (11). Our studies demonstrating that DNA synthesis occurs at ATP concentrations of $10^{-7}$-$10^{-6}$M in the presence of ARL6715 and an ATP-regenerating system, that UTP and ADP$\beta$S stimulate proliferation, and that suramin and cibacron blue 3GA inhibit ATP-induced fibroblast proliferation also suggest that P2Y receptors can initiate DNA synthesis in fibroblasts (10, 11). However, both agonist and antagonist data also suggest that P2X receptors can also mediate proliferative responses. The involvement of P2X receptors in the mitogenic response is in contrast to vascular SMC, however consistent with stimulation of proliferation through P2X receptors in osteoblasts (17). The data is also in agreement with previous reports demonstrating the presence of different subtypes of P2Y (P2Y1, P2Y2, P2Y4, P2Y6) and P2X (P2X3, P2X4, P2X7) receptors on human and rat fibroblasts (33, 34). The relative contribution of P2Y versus P2X receptor activation in hypoxia- and ATP-induced fibroblasts proliferation probably will be dependent on local ATP concentration and on relative expression level of P2Y and P2X receptors, which likely vary depending on the activation state of the fibroblast.

Little information exists regarding the post-receptor signaling pathways or transcription factors that are involved in ATP- or hypoxia-mediated fibroblast proliferation. Since many of the same conditions (oxidative stress, mechanical stretch, osmotic swelling) previously shown to cause increases in extracellular ATP also induce Egr-1 expression in cells, and because Egr-1 has been linked with cell proliferation, we evaluated the effect of ATP and hypoxia on Egr-1 expression in adventitial fibroblasts (24, 52, 53). ATP and hypoxia were both potent inducers of Egr-1 mRNA expression and exerted their effects over very similar time courses (Fig. 5, 6). In addition, both ATP and hypoxia increased the ability of Egr-1 to interact with a specific DNA recognition site (Fig. 9). EMSA revealed two proteins, which
specifically interact with consensus, but not with mutant, Egr-1 DNA probe. Supershift with specific Egr-1 antibodies revealed the inducible protein to be Egr-1. A second protein is not inducible and may represent a constitutively expressed transcription factor or co-regulator of Egr-1/DNA interaction. One candidate may be Sp-1, which has a DNA recognition site overlapping the site for Egr-1, which can be expressed in nuclei under basal conditions (23). Our observations are consistent with previous studies demonstrating that ATP can induce c-fos, c-jun, jun-B and Egr-1 in rat mesangial cells (54), and that hypoxia can induce Egr-1 in SMC and macrophages (55). Importantly, we found that P2 purinoceptor antagonists attenuated hypoxia-induced Egr-1 expression. Thus, Egr-1 appears to be an important hypoxia-regulated transcription factor whose activity can be regulated by P2 purinoceptors in vascular fibroblasts.

To further evaluate the role of ATP in hypoxia-induced growth, we evaluated signaling events upstream of Egr-1 and proliferation induced by ATP and hypoxia. We found that pertussis toxin (PTx) inhibited 40-50% of both ATP- and hypoxia-induced increases in Egr-1 mRNA expression and proliferation indicating that Gi/o proteins contribute nearly equally to hypoxia and ATP-induced responses in adventitial fibroblasts (Fig. 7, 8). The role of Gai/o proteins in hypoxia–induced proliferation previously has been established by us, and the fact that purinergic receptors can mediate some of their effects through PTx-sensitive Gai/o pathways has also been established (5). The data reported here continue to support the idea that Gai/o signaling is an important component of hypoxia-induced signaling and proliferation in fibroblasts. Of interest are observations in LLC-PK1 renal cells that Egr-1 upregulates transcription of the Gαi gene (52) that suggest that it will be important to determine if hypoxia creates a positive regulatory loop mediated through ATP to regulate Gαi gene expression in adventitial fibroblasts. P2 receptor antagonists and apyrase, again supporting a role for ATP release and purinoceptor activation in fibroblast growth, also attenuated hypoxia-induced increases in Egr-1 expression and ERK1/2 phosphorylation. In addition, our observations that ATP and hypoxia both act synergistically with peptide
growth factors operating through receptor tyrosine kinases also suggest that they use similar signaling pathways and are consistent with previous findings showing that GPCR and receptor tyrosine kinases (RTK) can act synergistically (56-58). Collectively, these observations are consistent with the idea that “constitutive” levels of ATP within the cellular environment might act as key determinants of the set point of many signal transduction pathways (18, 32).

Our data suggest that signaling pathways other than those mediated directly by Gαi may also contribute to Egr-1 mRNA expression and cell proliferation in response to ATP and hypoxia in adventitial fibroblasts. Other have demonstrated that P2Y receptor activation can be associated with stimulation of PTx insensitive G-protein coupled signaling, resulting in phospholipase C (PLC) activation (7, 36, 59-61). That Gαq and PLC may be important in hypoxia-induced proliferation is supported by observations showing that phosphatidylinositol-specific PLC is involved in hypoxia-induced DNA synthesis in bovine pulmonary artery adventitial fibroblasts (62). This would suggest that Gαq coupled PLC/PKC/Ca²⁺ signaling cascades may also be associated with hypoxia and ATP-induced Egr-1 expression and cell proliferation. In support of this are observations by Yan et al (55) and by Lo et al (63) who found that PKCβ2 and ERK1/2 are necessary for hypoxia-induced Egr-1 expression, although upstream signals were not defined. PI3-kinase/Akt signaling pathways have also been shown to be stimulated by hypoxia and ATP (64-66). It is also possible that P2X receptor stimulation under hypoxic conditions leads to increases in intracellular Ca²⁺ concentrations and stimulation of signaling pathways distinct from ERK1/2 and Egr-1. In support of this are our findings demonstrating that PPADS attenuates hypoxia-induced proliferation, but has little effect on ERK1/2 or Egr-1 activation. Future experiments will need to define the interactions between distinct signaling pathways, which are activated by hypoxia, and ATP that operate cooperatively to drive proliferative responses.
REFERENCES


ABBREVIATIONS

The abbreviations used are: Ado, adenosine; ADPβS, adenosine 5’-O-(2-thiodiphosphate); α,βmeATP, α,β-methylene adenosine 5’-triphosphate; ARL67156, 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine 5’-triphosphate; Bz-ATP, benzoilbenzoilATP 2’-3’-O-(4-benzoilbenzoil)adenosine 5’-triphosphate; MeS-ATP, 2-methylthioadenosine triphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2’,-4’-disulphonic acid; CSPD, Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5’-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl phenyl phosphate; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; Egr-1, early growth response-1 transcription factor; IGF-I, insulin-like growth factor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; PMA, phorbol 12-myristate13 acetate; ERK, extracellular signal-regulated kinase; MEK-1, ERK-activating kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; Tris, [tris(hydroxymethyl)aminomethane]; SDS, sodium dodecyl sulphate.
FIGURE LEGENDS

Figure 1. Hypoxia increases extracellular ATP concentration of fibroblasts and endothelial cells.
ATP concentration was measured in the media of cultured adventitial fibroblasts (A) and endothelial cells (B) that were exposed to normoxic (21% O₂) and hypoxic (3% O₂ and 1% O₂) conditions for varying periods of time. Following incubation, 1.5 ml aliquots of media were collected and prepared for ATP assays with a luciferase/luciferin kit. The results are expressed as mean ± standard error (* p<0.05 compared with normoxic (21% O₂) controls). Data illustrate one representative experiment for each cell type. Similar results have been obtained in a minimum of three individual experiments.

Figure 2. Chronic hypoxia delays the rate of extracellular ATP hydrolysis by fibroblasts and endothelial cells.
Panel A, Analysis of products of [α-³²P]-ATP hydrolysis by adventitial fibroblasts, chronically (14 days) cultured under normoxic (lines “−”) or hypoxic (3% O₂) conditions (lines “+”). To determine the rate of [α-³²P]-ATP hydrolysis, assays were carried out under both normoxic (21% O₂), (lines 1,3, 4, 6) or under hypoxic conditions (3% O₂) (lines 2, 5) in serum free media, containing 0.1 mM ATP, 10 mM MgCl₂, 1 mM EGTA and 5μCi of [α-³²P]-ATP. After indicated periods of time 2 μl- aliquot of extracellular media were spotted on PEI-cellulose plates and nucleotides were separated by thin layer chromatography in 1M LiCl. Panel B, Analysis of [α-³²P]-ATP hydrolysis by pulmonary artery endothelial cells, chronically (14 days) cultured under normoxic (lines “−”) or hypoxic (3% O₂) conditions (lines “+”). All assays were conducted under normoxic conditions; reaction mixtures and chromatography conditions were identical to those, described for adventitial fibroblasts. Columns “R” on Panels A and B represent [α-³²P]-ATP, incubated in DMEM without cells. Spots, corresponding to [α³²-P]-ATP, [α³²-P]-ADP and [α³²-P]-AMP were verified using unlabeled adenine nucleotide standards.
Figure 3. Effect of nucleotides, growth factors and hypoxia on DNA synthesis in adventitial fibroblasts.

Quiescent adventitial fibroblasts (72 hrs, 0.1% FBS-DMEM) were stimulated with the agonist of interest in the presence of 1 μCi of $[^3]$H-thymidine for 24 h and incorporated radioactivity was determined as described in “Materials and Methods”. Panel A: Effect of increasing concentrations of ATP on $[^3]$H-thymidine incorporation under normoxic (21% O$_2$) or hypoxic (3% O$_2$) conditions. Panel B: ATP-induced $[^3]$H-thymidine incorporation in the presence of ecto-ATPase inhibitor ARL67156 (300μM), or ATP-regeneration system (0.1 mg/ml creatine kinase, 4 mM phosphocreatine and 0.025 mg/ml myokinase). Panel C: Effect of hypoxia (3% O$_2$), ATP, UTP, ADP$_\alpha$S, BzATP (100 μM), MeSATP (300μM), $\alpha$,βmeATP (100 μM and 1000 μM), or adenosine (Ado, 100 μM) on $[^3]$H-thymidine incorporation in quiescent fibroblasts. Panel D: Effect of purified peptide mitogens PGDF (20 ng/ml), EGF (10 ng/ml) IGF-I (100 ng/ml) on $[^3]$H-thymidine incorporation under normoxic conditions and in the presence of ATP (100 μM) or hypoxia (3% O$_2$). The data represent the mean ± standard error (S.E.) from three to six independent experiments, conducted on distinct cell populations. (* p<0.05 compared with non-stimulated control).

Figure 4. Purinergic antagonists and apyrase attenuate ATP- and hypoxia-induced DNA synthesis in adventitial fibroblasts.

Cells were cultured under standard conditions and growth arrested for 72 hrs. Quiescent cells were preincubated with suramin (100 μM), cibacon blue 3GA (100 μM) for 30 min, PPADS (50 μM) for 60 min (Panel A), or with apyrase (5U/ml) for 2 hrs (Panel B) followed by stimulation with ATP (100 μM) or hypoxia (3% O$_2$) in the presence of 1μCi of $[^3]$H-thymidine/well for 24 h. At the end of incubation, $[^3]$H-thymidine incorporation was assessed as described in “Materials and Methods.” The quantitative data are expressed as percent increase over basal condition. The basal amount of $[^3]$H-thymidine incorporation was 1225 ± 140 cpm. Data are mean ± standard error (S.E.) from three to six independent
experiments (* p<0.05 compared with non-stimulated control (Panel A); ** p<0.05 compared with either ATP- or hypoxia-induced response (Panel B)).

**Figure 5. ATP and hypoxia induce Egr-1 mRNA expression in adventitial fibroblasts.**

Cells were plated at a density of 10 x10^3/cm^2, grown to 80% confluence and serum starved in 0.1% FBS/DMEM for 72 hrs. 15 µg of total cellular RNA, obtained from either control cells or cells stimulated with either 10 µM ATP, 10 µM UTP, hypoxia (3% O_2), hypoxia (3% O_2) + 10 µM ATP, 20 ng/ml PDGF, 10% fetal bovine serum (FBS), or 10 ng/ml PMA for 30 min were analyzed by Northern blot using digoxygenin-RNA probe for Egr-1 as described in “Materials and Methods”. To verify equivalent RNA loading and transfer, before hybridization membranes were stained with methylene blue. Experiments with the same agonists were repeated at least six times always giving similar results.

Figure 6. ATP and hypoxia induce Egr-1 mRNA expression in adventitial fibroblasts in a time- and dose-dependent manner.

Panel A. Effect of ATP on Egr-1 mRNA expression. Growth arrested cells were stimulated with 100 µM ATP for indicated periods of time (left), or stimulated with 10^{-9} - 10^{-3} M ATP for 1 h (right). Panel B. Effect of hypoxia on Egr-1 mRNA expression. Growth arrested cells were exposed to hypoxia (3% O_2) for 15 min-6 hrs (left), or exposed to indicated oxygen concentrations for 1 h (right). After treatment, total cellular RNA was extracted and 15 µg analyzed by Northern blot using Egr-1 digoxygenin-RNA probe. Egr-1 mRNA expression was normalized to β-actin mRNA or to 18S rRNA and expressed relative to the Egr-1 mRNA level in non-stimulated cells. Data represent average ± standard error (S.E.) of three independent experiments, performed on separate cell populations.

**Figure 7. Purinergic receptors, Gi proteins and Extracellular signal regulated kinase (ERK1/2) mediate both ATP- and hypoxia-induced Egr-1 mRNA expression.**
Growth arrested adventitial fibroblasts were treated with either PTx (100 ng/ml) for 20-24 h, PD 98059 (10 μM) for 1 h, suramin (100 μM) for 30 min at 37°C PPADS (100 μM) for 60 min, or with apyrase (5U/ml) for 2 hrs. After treatment, cells were stimulated with ATP (100 μM) or hypoxia (3% O₂) for 1 h, and then harvested for total RNA extraction. 15 μg of RNA was subjected to Northern blot analysis, and Egr-1 was identified using digoxygenin-RNA probe (Panel A, B, C). Before hybridization membranes were stained with methylene blue to verify equality of RNA loading and transfer. Bands, corresponding Egr-1 were normalized to 18S rRNA, and results were expressed relative to Egr-1 mRNA in non-stimulated cells. Data, shown in panel D represent average ± standard error (S.E.) from four to six independent experiments performed in four separate cell populations.

**Figure 8. Hypoxia and ATP induce ERK1/2 phosphorylation in Gi protein dependent manner.**

Adventitial fibroblasts were grown to 80% confluence and serum deprived for 72-94 hrs in DMEM/0.1% FBS. Panel A: After preincubation with PTx (100 ng/ml, 24h) cells were stimulated with either ATP (100 μM), or with hypoxia (3% O₂), for 10 min. Following stimulation, total cell lysates were prepared. Panel C and D: Quiescent cells were pretreated with purinergic antagonists (suramin, cibacron blue 3GA, PPADS) or with apyrase as described in Fig.7, and stimulated with either ATP (100 μM), or with hypoxia (3% O₂), for 10 min. Five μg of total cell protein was subjected to 10% SDS-polyacrylamide gel electrophoresis and Western blot analysis with antibodies against phosphorylated-ERK1/2 (phospho-Tyr202/Thr204) and non-phosphorylated ERK. Panel B and E: Phospho-ERK bands were normalized to non-phosphorylated ERK bands, and the quantitative data are expressed as a fold increases over basal stimulation. Data represent average ± standard error (S.E.) from four to six independent experiments.

**Figure 9. ATP and hypoxia induce Egr-1 protein expression and enhance its DNA binding activity.**

Panel A. Western blot analysis of Egr-1 protein in nuclear extracts of adventitial fibroblasts. Preconfluent cells were growth arrested in 0.1% FBS-DMEM for 72 h and then exposed to 100 μM ATP, or hypoxia
(3% O₂) in DMEM for indicated periods of time. At the end of each incubation, the nuclear fraction was isolated and 20 μg of nuclear protein was subjected to immunoblotting analysis with Egr-1 (C-19) rabbit polyclonal antibodies (See “Materials and Methods”). PMA (10 nM) was used as a positive control. Equality of protein loading was confirmed by staining of the membrane with Ponseau solution for each experiment. Panels B-D. Identification of Egr-1 DNA-binding activity in nuclear extracts of adventitial fibroblasts stimulated with ATP or hypoxia. Growth arrested adventitial fibroblasts were exposed to ATP (100 μM, 2 hrs) or hypoxia (3% O₂, 4 hrs) in fresh DMEM. At the end of incubation, nuclear extracts were isolated and 5 μg of protein were subjected to electrophoretic mobility shift assay (EMSA). Panel B: EMSA of nuclear extract with 32P-labeled DNA probe, containing Egr-1 binding consensus sequence, in the absence (-) or presence (+) of Egr-1 antibodies (Egr-1 Ab). Panel C: The same nuclear extracts were analyzed in the presence of excess of unlabelled Egr-1 binding DNA probe. Panel D: The same nuclear extracts were investigated for the ability to interact with 32P-labeled DNA probe, containing mutant Egr-1 binding sequence. Presented data were reproducible in minimum four individual experiments (* indicates unidentified specific binding complex, ** indicates nonspecific binding).

**Figure 10. A scheme showing how ATP might act as an autocrine/paracrine mediator of hypoxia-induced fibroblast proliferation.**

Hypoxia increases extracellular ATP level by, at least, two mechanisms: first, through the release of intracellular ATP across the plasma membrane, and second, by down-regulating ecto-nucleotidases (E1, E2 and E3), thus decreasing the rate of ATP hydrolysis. Both ATP and hypoxia stimulate adventitial fibroblast proliferation through G protein-coupled P2Y receptors, ionotropic P2X receptors and subsequent ERK1/2 and Egr-1-dependent signaling pathways. Additional possible mitogenic pathways are accompanied by question marks.
ACKNOWLEDGMENTS

We are indebted to Viktorya Marusyk for the excellent technical assistance and Dr. Aftab Ahmad for the helpful advices in the EMSA experiments. We thank Stephen Hofmeister and Marcia McGowan for the help in the preparation of the manuscript. We also wish to thank Dr. R. Nemenoff for his critical comments.
Fig. 1

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\[ \frac{\text{ATP}}{\text{Hypoxia}} \]

\[ \frac{\text{ATP}}{\text{Hypoxia}} \]

\[ \frac{\text{ATP}}{\text{Hypoxia}} \]

\[ \frac{\text{ATP}}{\text{Hypoxia}} \]
Fig. 5

<table>
<thead>
<tr>
<th>kb</th>
<th>Control</th>
<th>ATP</th>
<th>UTP</th>
<th>Hypoxia</th>
<th>Hypoxia + ATP</th>
<th>PDGF</th>
<th>FBS</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Egr-1
- 18S rRNA
Fig. 6

A

Egr-1 mRNA (fold of induction)

[min] 0 15 30 1 2 4 6 12 24

[ATP], M

0 10^-9 10^-8 10^-7 10^-6 10^-5 10^-4 10^-3
Fig. 6

B

Egr-1

![Image of Egr-1 mRNA expression](http://www.jbc.org)

**Egr-1 mRNA (fold of induction)**

<table>
<thead>
<tr>
<th>min</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
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<td>1.0</td>
<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
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**O₂ (%)**

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<tr>
<th>21</th>
<th>10</th>
<th>3</th>
<th>1</th>
<th>0</th>
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</thead>
<tbody>
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<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
<td>8.0</td>
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</table>
Fig. 7

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Egr-1</th>
<th>18S rRNA</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD 98059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + PTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + PD 98059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + Suramin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia + PTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia + PD 98059</td>
<td></td>
<td></td>
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<tr>
<td>Hypoxia + Suramin</td>
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<td></td>
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</table>

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Egr-1</th>
<th>18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPADS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + PPADS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia + PPADS</td>
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C

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<thead>
<tr>
<th>Condition</th>
<th>Egr-1</th>
<th>18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td></td>
</tr>
<tr>
<td>Apyrase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + Apyrase</td>
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<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia + Apyrase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Fig. 7**

Egr-1 mRNA (fold over basal)

- **Control**
- **ATP**
- **Hypoxia**

<table>
<thead>
<tr>
<th>Ptx</th>
<th>PD 98059</th>
<th>suramin</th>
<th>PPADS</th>
<th>Apyrase</th>
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<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Downloaded from [http://www.jbc.org/](http://www.jbc.org/) by guest on November 17, 2017
Fig. 8

A

ATP
- + - + - + - +

Phospho-ERK-1/2
- Ptx + Ptx - Ptx + Ptx

Total ERK-1/2
- Ptx + Ptx - Ptx + Ptx

21% O₂ 3% O₂

B

Phospho-ERK-1/2 (fold over basal)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Ptx</td>
<td>- Ptx</td>
<td>+ Ptx</td>
</tr>
<tr>
<td>21% O₂</td>
<td>2%</td>
<td>6%</td>
</tr>
<tr>
<td>3% O₂</td>
<td>2%</td>
<td>6%</td>
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</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on November 17, 2017
Fig. 8

C

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>ATP</th>
<th>3% O2</th>
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<tbody>
<tr>
<td>Phospho-ERK-1/2</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>Total ERK-1/2</td>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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<tr>
<td>Suramin</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>Cibacron blue</td>
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<td>-</td>
<td>+</td>
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D

<table>
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<tr>
<th></th>
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<th>3% O2</th>
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<tr>
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<td>Total ERK-1/2</td>
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<tr>
<td>Apyrase</td>
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<td>-</td>
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</table>

E

![Bar chart](chart.png)
Fig. 9

A

Egr-1

Time (hrs) 0 2 4 6 24 4

ATP (100μM) PMA

Hypoxia (3% O₂) kDa

97

66
Fig. 9

B

<table>
<thead>
<tr>
<th>control (cont)</th>
<th>ATP</th>
<th>3%O₂</th>
<th>PMA</th>
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</thead>
<tbody>
<tr>
<td>supershift</td>
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<tr>
<td>bound Egr-1</td>
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</table>

C

<table>
<thead>
<tr>
<th>control (cont)</th>
<th>ATP</th>
<th>3%O₂</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>bound Egr-1</td>
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<tr>
<td>bound Egr-1</td>
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D

<table>
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<tr>
<th>control (cont)</th>
<th>ATP</th>
<th>3%O₂</th>
<th>Free probe</th>
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<tbody>
<tr>
<td></td>
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<td>unlabeled Egr-1 oligo</td>
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<tr>
<td></td>
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<td>mutant 32P-Egr-1 oligo</td>
</tr>
</tbody>
</table>

Egr-1 Ab

- + - + - + - +
Fig. 10

Hypoxia

(Inhibition of ATP hydrolysis)

ATP → ADP → AMP → Ado

(P2Y) E1 E2 E3

G proteins

ERK-1/2

Egr-1

proliferation

(Increase of extracellular ATP)
Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth: signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor

Evgenia V. Gerasimovskaya, Shama Ahmad, Carl W. White, Peter L. Jones, Todd C. Carpenter and Kurt R. Stenmark

*J. Biol. Chem.* published online September 18, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203012200

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