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Placenta

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Hypoxia is an effective stimulus for vesicular release of ATP from human umbilical vein endothelial cells

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A B S T R A C T

Introduction: Hypoxia induces dilatation of the umbilical vein by releasing autocoids from endothelium; prostaglandins (PGs), adenosine and nitric oxide (NO) have been implicated. ATP is vasoactive, thus we tested whether hypoxia releases ATP from primary Human Umbilical Vein Endothelial Cells (HUVEC).
Methods: HUVEC were grown on inserts under no-flow conditions. ATP was assayed by luciferin–luciferase and visualised by quinacrine labeling. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was imaged with Fura-2.
Results: ATP release occurred constitutively and was increased by hypoxia (PO_2: 150–8 mmHg), ~10-fold more from apical, than basolateral surface. Constitutive ATP release was decreased, while hypoxia-induced release was abolished by brefeldin or monensin A, inhibitors of vesicular transport, and LY294002 or Y27632, inhibitors of phosphoinositide 3-kinases (PI_3K) and Rho-associated protein kinase (ROCK). ATP release was unaffected by NO donor, but increased by calcium ionophore, by >60-fold from apical, but <25% from basolateral surface. Hypoxia induced a small increase in $[Ca^{2+}]_i$ compared with ATP (10 μM); hypoxia inhibited the ATP response. Quinacrine-ATP fluorescent loci in the perinuclear space, were diminished by hypoxia and monensin, whereas brefeldin A increased fluorescence intensity, consistent with inhibition of anterograde transport.
Discussion.: Hypoxia within the physiological range releases ATP from HUVEC, particularly from apical/adluminal surfaces by exocytosis, via an increase in $[Ca^{2+}]_i$, PI_3K and ROCK, independently of NO. We propose that hypoxia releases ATP at concentrations sufficient to induce umbilical vein dilation via PGs and NO and improve fetal blood flow, but curbs amplification of ATP release by autocrine actions of ATP, so limiting its pro-inflammatory effects.

1. Introduction

The umbilical vein, which is critically important in the delivery of oxygen to the fetus, is regulated by autocoids, rather than autonomic nerves [1]. At normal levels of oxygenation, it has vasoconstrictor tone: falls in partial pressure of oxygen (PO_2) induce umbilical venous dilation, whereas rises in PO_2 induce vasoconstriction [2]. Hypoxia-induced dilation has been attributed to endothelium-dependent release of NO and hyperoxia-induced vasoconstriction, to endothelin [3]. Adenosine and ATP have also been implicated. Adenosine is released from the placenta into umbilical venous blood in anemia, hypoxia, growth retardation and pre-eclampsia [4–6]. Moreover, hypoxia decreases adenosine uptake via its endonucleoside transporter (ENT-1) in human umbilical

vein endothelial cells (HUVEC) [6]. Adenosine dilates umbilical vein by stimulating A_{2A} receptors to increase NO synthesis [7].

ATP is also released into placental circulation in normal pregnancy, while high ATP concentrations in pre-eclampsia are considered a danger signal [5]. ATP is rapidly metabolised by ectonucleotidases to adenosine, but ATP itself is vasoactive. ATP evokes vasoconstriction in placenta via P2X receptors on vascular smooth muscle, but induces vasodilation via P2Y receptors on HUVEC, by increasing NO and PGI_2 synthesis [8–10]. Further, the balance of ATP, ADP and adenosine regulate platelet aggregation, inflammation, immunity, and vascular proliferation, high concentrations of ATP being pro-inflammatory and immunostimulatory [5,10–12]. It has been reported that ATP is released from endothelial cells by hypoxia [1,4,5]. However, whilst hypoxia augmented shear stress-stimulated ATP release from primary HUVEC, hypoxia alone did *not* affect ATP release [13]. But, in that study, O_2 was decreased from 95 to $0\%O_2$, which may well have masked the effects of hypoxia over the physiological range. Decreasing O_2 from 20

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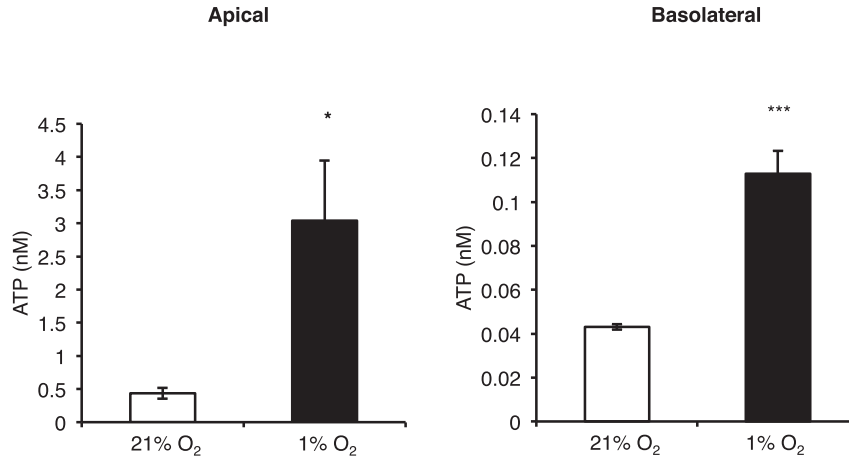 E-mail address: j.m.marshall@bham.ac.uk (J.M. Marshall).

Apical

Basolateral

Fig. 1. Effect of hypoxia on ATP release from apical and basolateral surfaces of HUVEC. Columns show mean ± SEM; *, ***: hypoxia vs normoxia; $P < 0.05$, <0.01 respectively ($n = 9$, $N = 3$ in each case; n: number of inserts, N: number of donors). Note different scales for ordinates.

to 1% did release ATP from pulmonary artery vasa vasorum endothelial cells (VVEC). However, these cells were obtained from chronically hypoxic calves, cultured to passages 2–7 and growth arrested [14]: they cannot be compared to normally proliferating primary HUVEC. Further, in human endothelial cell lines, hypoxia (2%O_2) *inhibited* ATP release via connexion 43 (Cx43) hemichannels [15].

Thus, our primary hypothesis was that hypoxia over the physiological range releases ATP from primary HUVEC, predominantly from apical rather than basolateral surfaces; we considered this would give maximum potential for ATP to influence blood cell interaction and vascular regulation. Since our results supported this hypothesis, we hypothesised that hypoxia-induced release of ATP is vesicular, given release of ATP from HUVEC by shear stress, and hypoxia-induced release from VVEC were ascribed to exocytosis [14–16]. However, ATP can be released by various transporters and channels [17]. Notably, thrombin-induced ATP release from HUVEC and hypoxia-induced ATP release from erythrocytes were attributed to pannexin channels, which are *inhibited* by NO [18–20]. But, both NO and hypoxia increased *adenosine* release from endothelial cells, which we attributed to NO out-competing O_2 for their binding site on cytochrome oxidase and decreasing ATP synthesis [21,22].
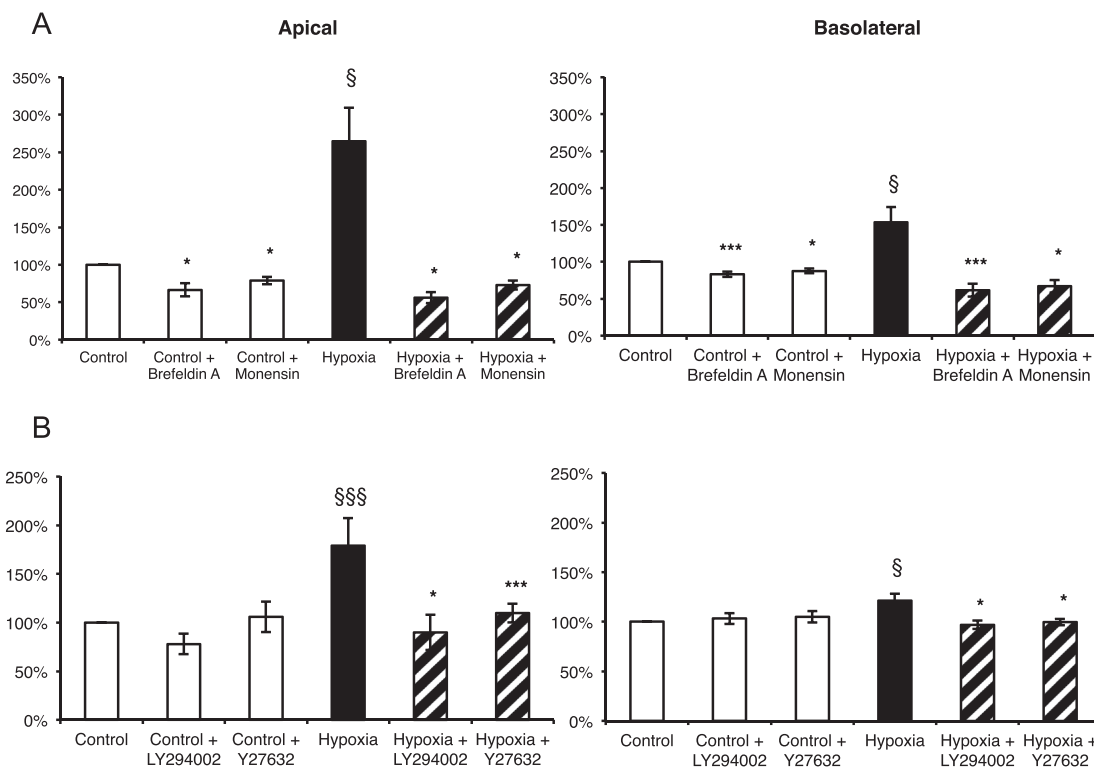

Fig. 2. Effect of pharmacological antagonists on constitutive and hypoxia-induced ATP release from HUVEC. Effects of vesicular transport inhibition with brefeldin A or monensin (A), and effects of PI$_3$K and ROCK inhibition with LY294002 or Y27632 (B). In each case, apical: left, basolateral: right. Hypoxia-induced ATP release in absence and presence of inhibitors shown in black and hatched columns respectively. All data are shown as % of constitutive release in normoxia (mean ± SEM). §, §§§: hypoxia vs normoxia; $P < 0.05$, $P < 0.001$ respectively, *, ***: vs normoxic or hypoxic control; $P < 0.05$, 0.01 respectively ($n = 18$, $N = 6$ in each case; n = number of inserts, N = number of donors).
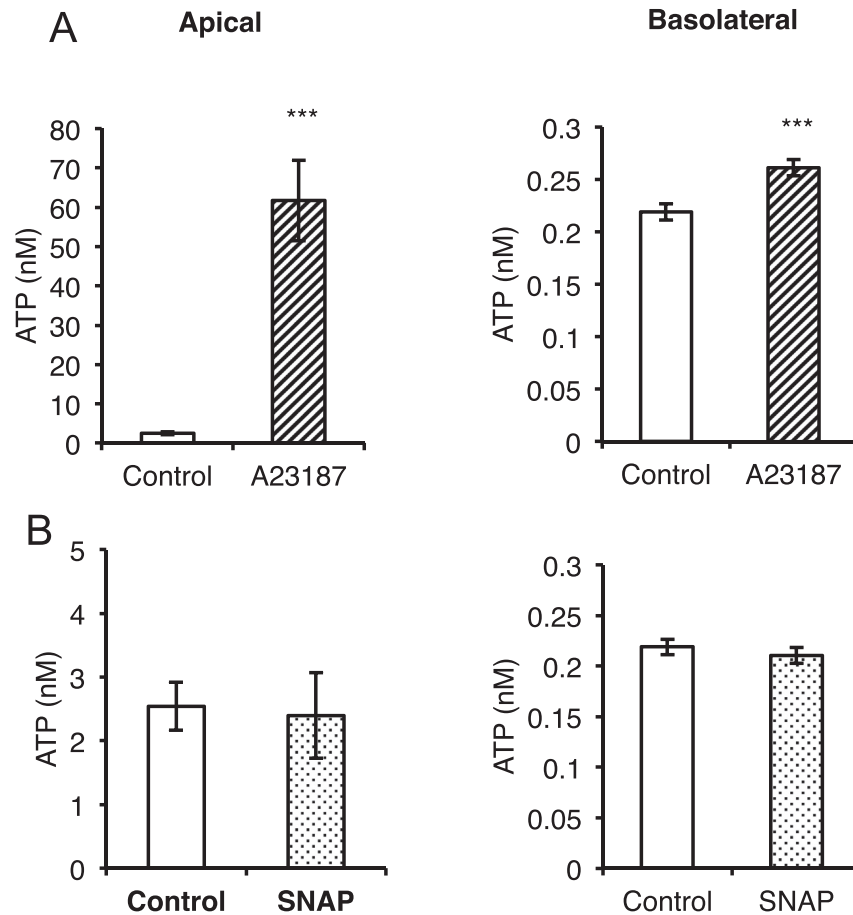
Fig. 3. Effect of Ca^{2+} ionophore A23187 (top) and NO donor (bottom) on ATP release from apical and basolateral surfaces of HUVEC in normoxia. Columns show mean ± SEM. *** vs control; $P < 0.01$ ($n = 9$, $N = 3$; n = number of inserts, N = number of donors). Note different scales for ordinates showing apical (left) and basolateral (right) release of ATP.

However, it could be that NO actually releases ATP, which is metabolised extracellularly to adenosine. Thus, we hypothesised that NO releases ATP from HUVEC.

2. Methods

Umbilical cords were obtained with informed consent (West Midlands-South Birmingham NHS Regional Ethics Committee) from 25 normal, full-term pregnancies, and isolated as described previously [23]. For a detailed account of methodology see on-line data Supplement.

2.1. ATP release

First passage HUVEC were seeded onto 24-well culture inserts, to allow differentiation of release from apical and basolateral surfaces [24]. After monolayer formation, they were incubated at 37 °C with 5% CO_2 (normoxia), or 1% O_2/5% CO_2 in N_2 (hypoxia) for 30 min. Medium was then removed from apical and basolateral compartments for ATP assay by conventional luciferin–luciferase assay.

To assess effects of vesicular transport inhibition, HUVEC were pre-incubated for 60 min with brefeldin A (20 μM), monensin (10 μM) or vehicle (1:1000 DMSO). The roles of phosphoinositide 3-kinases (PI3K) or Rho-associated protein kinase (ROCK), were assessed by using LY294002 (20 μM), or Y27632 (10 μM), that inhibit PI3K and ROCK respectively [14,25,26]. To examine effects of increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), or NO donor, medium was replaced with one containing ionophore A23187 (10 μM), NO donor S-Nitroso-N-acetylpenicillamin (SNAP; 100 μM), or vehicle (1:1000 DMSO).

2.2. $[Ca^{2+}]_i$ imaging

HUVEC were loaded with Fura-2 AM (12.5 μM), placed in a sealed cuvette and perfused with Krebs' at 37 °C bubbled with 95%air/5%CO_2 (normoxia), or 95%N_2/5% CO_2 (hypoxia): outflow PO_2 was 147–153 and 7.6–9.9 mmHg respectively.

On an inverted microscope, Fura-2 AM was excited alternately at 340 and 380 nm and emissions captured at 510 nm with a CCD camera. Images were analysed offline to quantify changes in $[Ca^{2+}]_i$. Initial experiments on single cells showed calcium responses in different cells were synchronised, thus all cells within the area of interest were analysed as one unit.

Dose-responses curves were obtained by adding 1, 10, 100, 300 or 1000 μM ATP. In HUVEC from 6 donors we verified the $[Ca^{2+}]_i$ response to ATP was mediated via P2 receptors, by challenging with ATP (10 μM) after suramin (100 μM). The effect of hypoxia was tested by switching from normoxia to hypoxia for 4 min. The effect of ATP (10 μM) in hypoxia was also tested.

2.3. ATP visualisation

HUVEC seeded on glass coverslips were incubated for 60 min at 37 °C with quinacrine (0.5 μM) which labels ATP [16,27,28]. DAPI was applied to stain nuclei and cells were examined with a confocal microscope.

HUVEC were incubated in either normoxia or hypoxia for 60 min and then viewed with UV epi-illumination (excitation at 494 nm, emission captured at 518 nm) or bright field illumination. In addition, HUVEC were incubated in brefeldin A (10 μM), monensin (100 μM), or vehicle (1:1000 DMSO) for 60 min, labeling with quinacrine (30 μM) for a further 5 min. All comparisons were made between cells of the same donor, at identical exposure time and gain settings.

2.4. Statistical analysis

Data are expressed as mean ± S.E.M. Comparisons were made by using Student's unpaired t-test, or repeated measures ANOVA and post hoc Fisher PLSD tests were used to compare effects of different pharmacological agents and their vehicle. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of hypoxia on ATP release from HUVEC

In normoxia, HUVEC constitutively released ATP from apical and basolateral surfaces ($P < 0.05$ or 0.001: Fig. 1). Allowing for the
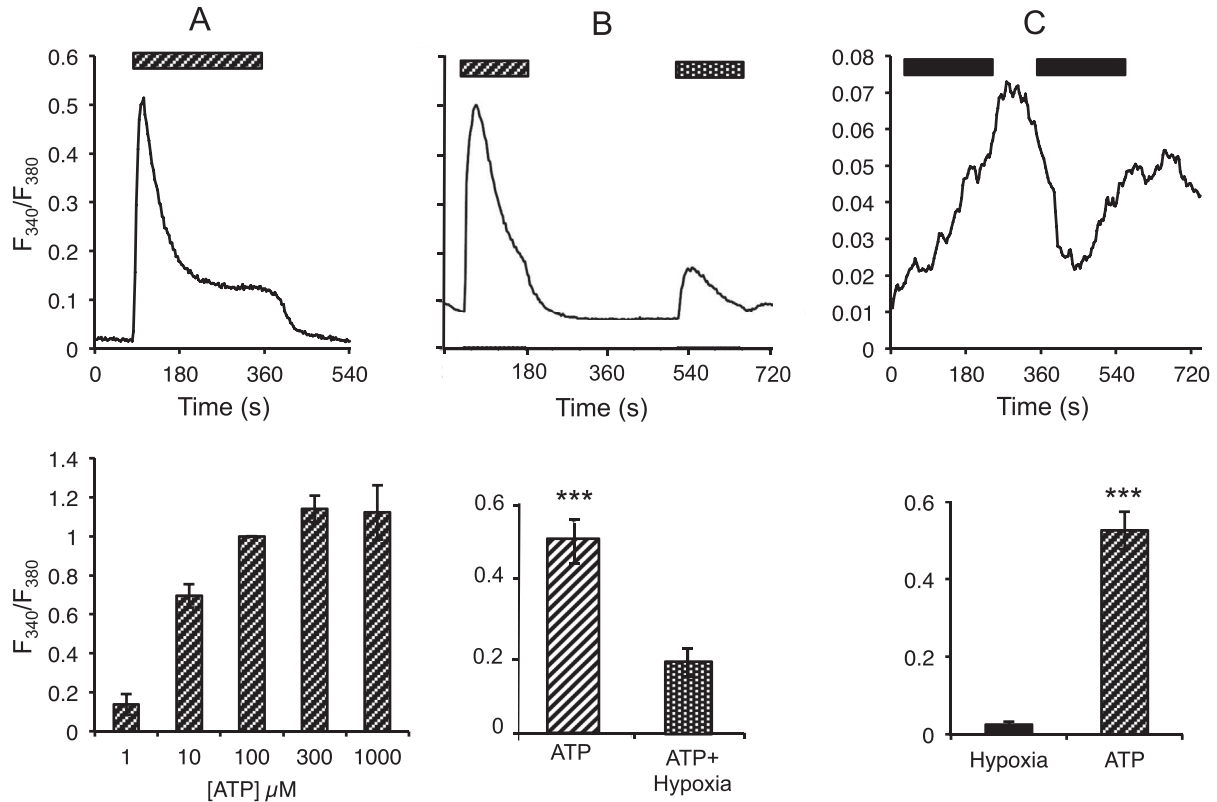
Fig. 4. Changes in $[Ca^{2+}]_i$ evoked in HUVEC by ATP in normoxia and acute hypoxia, and by hypoxia. Top: original recordings of $[Ca^{2+}]_i$ in HUVEC in response to ATP (10 μM) in normoxia (A), ATP (10 μM) in normoxia vs hypoxia (B), hypoxia (C), note different scale for ordinate. Bottom: dose response relationship for ATP-induced increase in $[Ca^{2+}]_i$ relative to that evoked by 100 μM ATP (A), mean change in $[Ca^{2+}]_i$ evoked by ATP (10 μM) in normoxia vs hypoxia (B), mean change in $[Ca^{2+}]_i$ evoked by hypoxia compared with that evoked by ATP (10 μM, C). Columns show mean ± SEM (5 or 6 samples from 6 different donors in each case). ***: $P < 0.01$, ATP in normoxia vs ATP in hypoxia, or vs hypoxia.

volume of apical and basolateral compartments (200; 700 μl respectively), apical release was ~5-fold greater than basolateral release. Hypoxia increased ATP release by ~6-fold and 3-fold from apical and basolateral surfaces respectively, absolute amounts being ~10-fold greater from apical surface.

Vesicular transport inhibition with brefeldin A, or monensin decreased constitutive release ($P < 0.05$) and abolished hypoxia-induced release of ATP from apical and basolateral surfaces ($P < 0.05$ or 0.001: Fig. 2A). Inhibition of PI3K and ROCK with LY294002 or Y27632 did not affect constitutive ATP release, but abolished hypoxia-induced ATP release from both surfaces ($P < 0.05$ or 0.001: Fig. 2B). Vehicle did not affect ATP release (data not shown).

Ca^{2+} ionophore A23187 increased ATP release far more from apical than basolateral surface (Fig. 3A). The NO donor SNAP did not affect apical or basolateral release (Fig. 3B).

3.2. $[Ca^{2+}]_i$ imaging

ATP evoked a rapid increase in $[Ca^{2+}]_i$ in HUVEC, followed by a sustained phase; the $[Ca^{2+}]_i$ elevation evoked by ATP was concentration-dependent (Fig. 4A). After suramin, the $[Ca^{2+}]_i$ response to ATP (10 μM), was slower and maximum $[Ca^{2+}]_i$ elevation was inhibited by 57.8 ± 5.8% (data not shown).

In hypoxia, the increase in $[Ca^{2+}]_i$ evoked by 10 μM ATP was blunted relative to that evoked in normoxia (Fig. 4B). Hypoxia alone caused a small, reversible increase in $[Ca^{2+}]_i$ that was repeatable; this response was much smaller than that evoked by ATP (10 μM; Fig. 4C).

3.3. Visualisation of ATP

Confocal imaging revealed a punctate pattern of ATP labeled with quinacrine, clusters of highest fluorescence were concentrated in perinuclear space, but fluorescent loci also occurred at the cell boundary. Images at 1.0 μm intervals in the z plane showed HUVEC monolayers were ~4.0 μm thick; each fluorescent locus was 0.5—1.4 μm diameter (Fig. 5A).

Imaging by fluorescence and bright field microscopy confirmed high intensity fluorescent loci, mainly in perinuclear space (Fig. 5B). Real-time observation revealed fluorescent loci were not stable: an occasional non-recurring transient increase in fluorescence intensity occurred at a particular locus followed by rapid disappearance. In hypoxia, quinacrine-staining was markedly attenuated, fluorescence intensity of most loci being much lower (Fig. 5C).

Brefeldin A *increased* the number and intensity of fluorescent areas especially in perinuclear space (Fig. 6B vs A). By contrast, monensin *decreased* the number and intensity of fluorescent loci (Fig. 6C vs A).

4. Discussion

In the present study on primary HUVEC, we observed quinacrine-labeled fluorescent loci mainly in the perinuclear region, as described for ATP-containing vesicles in HUVEC, presumably in the region of Golgi apparatus [16,17,27]. They were ~0.5—1.4 μm diameter and similar in size to ATP-containing vesicles labeled with quinacrine in liver and pancreatic acinar cells [27,28]. We confirmed that monensin, which facilitates Na^+/H^+ transport and collapses membrane potential across vesicles [29], greatly
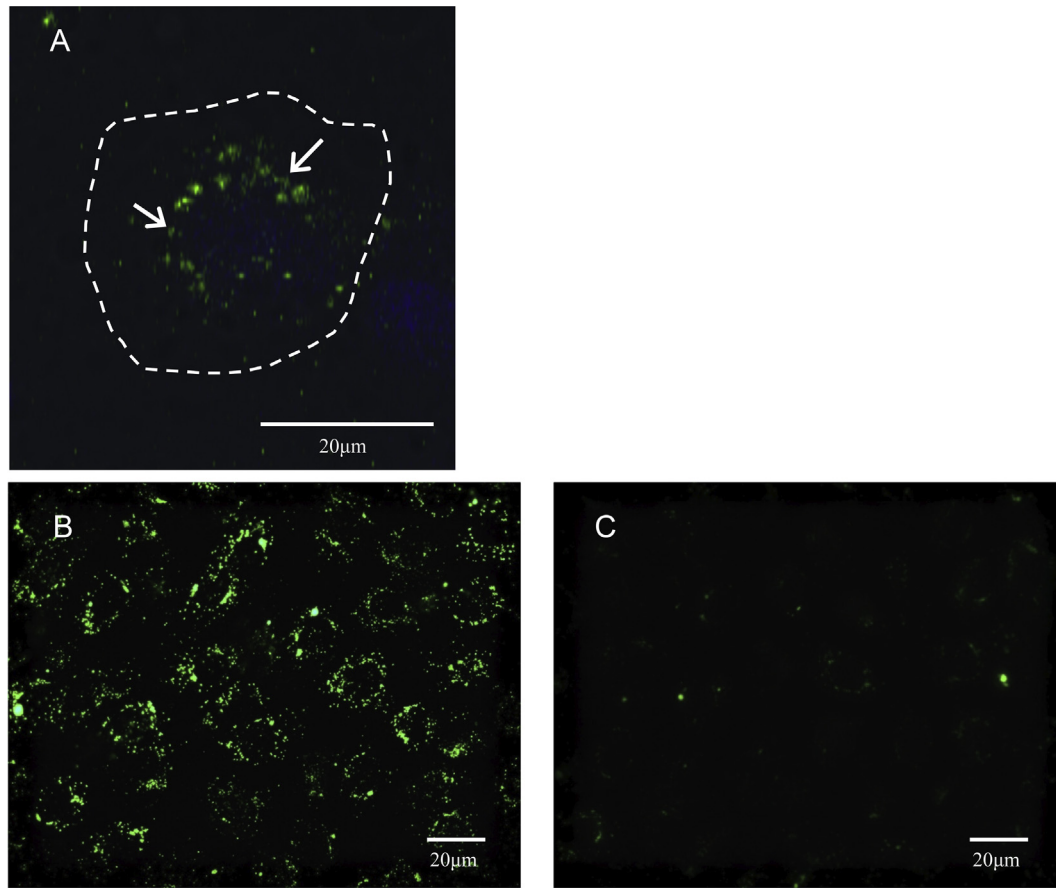
Fig. 5. Images of HUVEC showing quinacrine-staining of ATP in normoxia and hypoxia. A: example of confocal image of HUVEC monolayer taken at 1 μm intervals, dual-labeled with quinacrine and DAPI. Dashed line drawn around cell boundary. Nucleus appears blue; ATP loci green (white arrows) in perinuclear region (Image representative of 4 samples from 2 donors) Below: fluorescent images of HUVEC stained with quinacrine before (B) and after (C) exposure to hypoxia (representative of 3 samples from 2 donors, both in normoxia and hypoxia).

decreased fluorescent intensity in HUVEC [16]. This is consistent with dispersion of ATP from vesicles. That brefeldin A *increased* the intensity of fluorescence in the perinuclear region is consistent with it inhibiting anterograde transport and facilitating retrograde transport such that ATP accumulated in the mixed Golgi/endoplasmic reticulum system [30].

4.1. Constitutive ATP release

The spontaneous bursts of high intensity fluorescence we observed at discrete loci in HUVEC seemed similar to those described in astrocytes, liver, bronchial and lung epithelial cells [28,31,32], while our assays demonstrating constitutive release of ATP that was much greater from apical, than basolateral surfaces, were consistent with experiments on a range of endothelial cells including HUVEC [24]. Our novel finding that this constitutive release was decreased by brefeldin A and monensin, strongly suggests that primary HUVEC, under no-flow conditions at PO_2 ~150 mmHg, occasionally discharge ATP by exocytosis, particularly from apical surfaces. We cannot exclude spontaneous release of ATP through Cx-43 hemi-channels [32], or by H^+-ATP synthase that generated ATP under basal conditions in HUVEC [17,33] and were localised in apical membranes.

It is very unlikely constitutive ATP release was through pannexin channels, implicated in agonist-induced ATP release from HUVEC [19]. For NO donor did not inhibit constitutive ATP release, contrasting with the finding that NO nitrosylates pannexin channels, so

inhibiting ATP release [20]. That NO did not *increase* ATP release from HUVEC nicely differentiates the mechanisms for ATP and adenosine release in hypoxia. We concluded that increased NO, or hypoxia rebalances the competitive interaction between NO and O_2 on cytochrome oxidase in endothelial cells, so leading to adenosine release [21,22]. The present evidence agrees with that conclusion. There is no reason to suppose NO releases ATP, which is metabolised extracellularly to adenosine.

4.2. Hypoxia-induced ATP release

Our findings that hypoxia greatly stimulated ATP release from the apical surface, that this was abolished by brefeldin A or monensin and that hypoxia considerably decreased the intensity of fluorescent loci, indicate that hypoxia-induced ATP release from primary HUVEC is mainly attributable to exocytosis. This conclusion directly contrasts with the report that in primary HUVEC, hypoxia *per se* had *no* effect on ATP release [13]. However, "normoxia" in that study was equilibration with 95%O_2 for 1 h [13], which generated a 2–3 fold increase in reactive oxygen species (ROS) and $[Ca^{2+}]_i$ in endothelial cells [34]. This may have overwhelmed the ability of "hypoxia" (0%O_2) to evoke exocytotic ATP release if this response is dependent on a small increase in ROS and $[Ca^{2+}]_i$ ([35], see below). Irrespective, ours is the first study to demonstrate that a fall in PO_2 from ~150 to ~9 mmHg — over the physiological range — releases ATP from HUVEC by exocytosis, predominantly from apical surfaces.
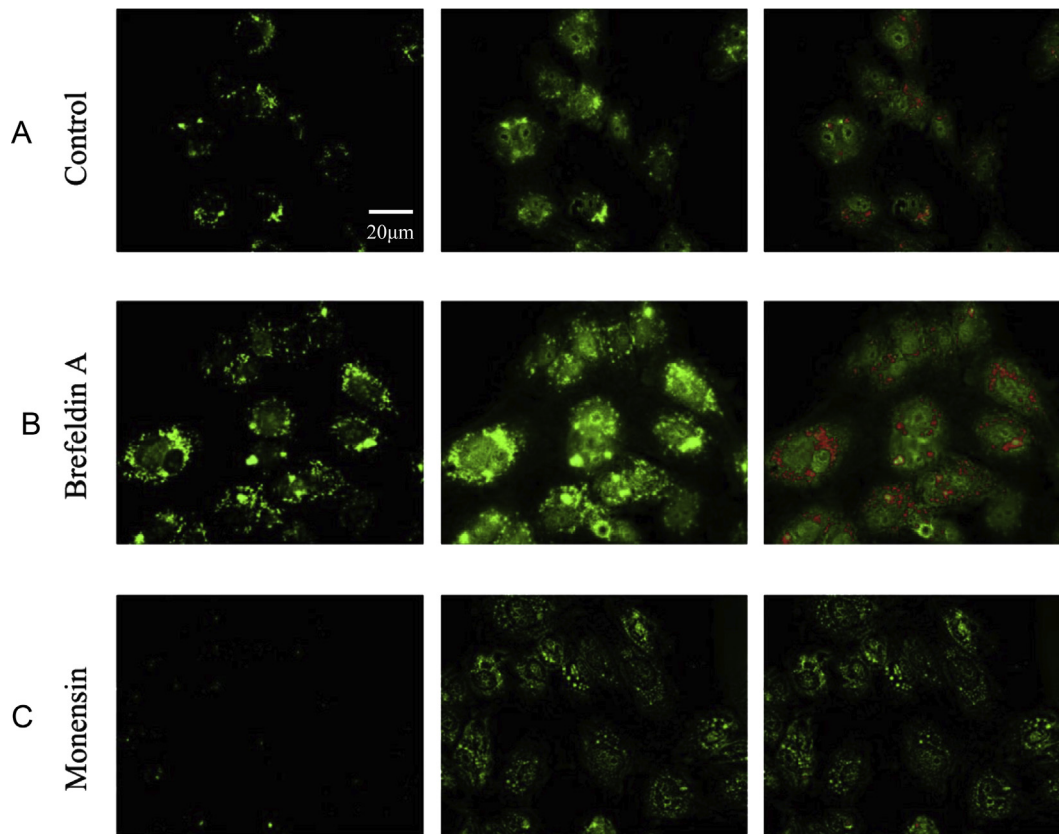
Fig. 6. Effects of brefeldin A and monensin on quinacrine-stained HUVEC. Fluorescent and bright field images of quinacrine-stained HUVEC with vehicle (A), after brefeldin A (B), after monensin (C). Left; epi-illumination showing quinacrine-induced fluorescence, middle; epi-illumination + bright-field trans-illumination, right; overlay of quinacrine fluorescence (pseudo-colored in red) over bright-field image. Brefeldin A led to accumulation of quinacrine fluorescence in perinuclear region, whereas monensin led to loss of quinacrine fluorescence. Representative in each case of 3 samples from 2 donors.

That ATP release was polarised in normoxia and hypoxia, raises the question of whether the mechanisms that stimulate hypoxia-induced ATP release are polarised, or ATP release itself. Since Ca^{2+} ionophore evoked a 30-fold increase in ATP release from the apical surface, but only a ~20% increase from the basolateral surface, we propose that vesicles primed to release ATP are preferentially located near the apical surface and are triggered to discharge ATP by an increase in $[Ca^{2+}]_i$ [36].

That hypoxia evoked a small increase in $[Ca^{2+}]_i$ is consistent with this being integral to hypoxia-induced ATP exocytosis. The hypoxia-evoked increase in $[Ca^{2+}]_i$ was much smaller than that evoked by 10 μM ATP which is comparable to the finding in human saphenous vein endothelial cells [35]. This disparity suggests that ATP released by hypoxia is unlikely to have acted back on P2 receptors to further increase $[Ca^{2+}]_i$ and augment ATP release, as shown in aortic endothelium and HUVEC during hypotonic cell swelling [37,38]. In fact, we showed that hypoxia *blunted* the increase in $[Ca^{2+}]_i$ evoked by exogenous ATP. This may be explained by cross-talk between signaling pathways for hypoxia and ATP. In saphenous vein endothelial cells, hypoxia-induced release of ROS from mitochondria acted on ryanodine receptors to release Ca^{2+} from intracellular stores, whereas ATP stimulated P2 receptors to release Ca^{2+} via inositol triphosphate receptors, which were *inhibited* by ROS [35]. Thus, ROS generated by hypoxia may inhibit ATP-evoked Ca^{2+} release. Functionally, our results raise the possibility that the inhibitory effect of hypoxia on ATP-evoked increases in $[Ca^{2+}]_i$ helps prevent self-regenerating release of ATP from HUVEC and its potential harmful effects [5]. A similar suggestion was made for the inhibitory effect of NO synthesised by

ATP acting on P2Y receptors, on ATP release through pannexin channels [20].

Inhibition of PI$_3$K and ROCK with LY294002 and Y27632 respectively, abolished hypoxia-induced ATP release from HUVEC. Similarly, PI$_3$K inhibition abolished hypotonic swelling-induced ATP release from liver cells, while PI$_3$K and Rho-ROCK inhibition attenuated hypoxia-induced ATP release from growth arrested, chronically hypoxic VVEC; both were attributed to exocytosis [14,28]. Importantly, hypoxia activated PI$_3$K in coronary artery endothelial cells [39] and increased RhoA and ROCK expressions in HUVEC [40]. Further, PI$_3$K was implicated in vesicular transport, while RhoA-ROCK signaling was implicated in actin re-organisation during hypotonic swelling [37,38]. Thus, we conclude that vesicular release of ATP from primary HUVEC in acute hypoxia involves an increase in $[Ca^{2+}]_i$ and is dependent on a PI$_3$K, Rho-ROCK pathway.

We assayed ATP at 30 min of hypoxia and did not establish the time course of release. Hypotonic swelling stimulated ATP release within 10 min, and ATP accumulated gradually until 30 min [37,38]. Increased shear stress released ATP from HUVEC within 1–2 min [13,16] and we showed hypoxia increased $[Ca^{2+}]_i$ within 2 min. Thus, it seems likely ATP release began within 2 min of hypoxia and that medium [ATP] gradually increased. This should be tested.

At 30 min of hypoxia, medium [ATP] reflects the balance between that released into the medium and its metabolism by ectonucleotidases. Vesicular release is directly temperature-dependent below 24 °C, but ectonucleotidase activity is much greater at 37 °C than 24 °C [24]. Thus, our experiments at 37 °C may have facilitated vesicular release of ATP, but enhanced adenosine formation relative to studies at room temperature [24,37,38].

Irrespective, it may be noted that [ATP] measured in hypoxia was in the nM range, whereas that required to evoke an increase in $[Ca^{2+}]_i$ was in the μM range. This disparity is consistent with [ATP] measured in medium of HUVEC during shear stress and hypotonic swelling [14,37,38], and with [ATP] required in HUVEC to stimulate NO and PGI_2 synthesis and vasodilation via P2Y receptors [8–10]. By contrast, [ATP] close to the cell surface and P2 receptors, is in the μM range when ATP is released [27,41] and much higher than measured in medium in vitro, or plasma in vivo [42,43]. Thus, our findings are compatible with current views that locally-released ATP and adenosine play important roles in vascular regulation [10]. Not only does hypoxia inhibit adenosine uptake into HUVEC via ENT-1, but ATP also inhibits ENT-1 transport by acting on P2Y receptors [6,44]. Thus, hypoxia-induced ATP release from HUVEC would be expected to increase extracellular concentrations of both ATP and adenosine.

In summary, the present results show that acute hypoxia is an effective stimulus for ATP release from primary HUVEC, primarily from the apical surface, i.e. towards the blood stream in vivo. We show that ATP is released from vesicles located predominantly at the apical surface, by exocytosis, involving an increase in $[Ca^{2+}]_i$ and depending on PI_3K, ROCK signaling. The ability of ATP acting on P2 receptors to increase $[Ca^{2+}]_i$ was inhibited by hypoxia, suggesting that self-regenerating release of ATP is blunted by hypoxia; this may limit pro-inflammatory and pro-thrombotic effects of high ATP concentrations in umbilical vein [5,12,13]. Nevertheless, a fall in PO_2 from ~100 to 8.5 mmHg induced near maximal dilation of human umbilical vein that was endothelium-dependent [2,3]. We suggest ATP is released in adequate concentrations from HUVEC during hypoxia to stimulate P2Y receptors to increase NO and PGI_2 synthesis [9,10], thereby contributing to umbilical vein dilation and increased fetal blood flow reported, for example, during fetal respiratory movements associated with hypoxia [45]. Adenosine generated from ATP by ectonucleotidases in hypoxia may augment venodilation, but limit thrombosis and inflammation [7,11,12].

Conflict of interest

None

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2015.04.005.

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