Functional characterization of HUVEC-CS: Ca^{2+} signaling, ERK 1/2 activation, mitogenesis and vasodilator production

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Abstract

While many endothelial cell lines exist, few are of human origin with characteristics close to the parent endothelial cell. We derived a subline (HUVEC-CS) of immortalized human umbilical vein endothelial cells (HUVEC-C) that proliferate in standard growth media and exhibit positive acetylated low-density lipoprotein (AcLDL) uptake, express eNOS, CD31 and ve-cadherin, and spontaneously form capillary-like structures when grown on Matrigel. HUVEC-CS also maintain endothelial cell characteristics at the level of mitogenesis, kinase activation and vasodilator production. Like primary HUVEC cells, HUVEC-CS express many of the key proteins necessary for vasodilator production, including epithelial nitric oxide synthase (eNOS), HSP 90, cav-1 and -2, $cPLA_2$, and COX-1 and -2. Prostaglandin I synthase (PGIS) was not detectable by Western blot analysis, consistent with primary HUVEC in which PGI_2 production is minimal. Receptors were detected for angiotensin II (AII), bradykinin, ATP and growth factors. ATP induced a dose- and time-dependent rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Initially, ATP stimulates P_2Y receptors rather than P_2X receptors, as demonstrated by the inability of ATP to initiate a Ca^{2+} response subsequent to emptying of the internal Ca^{2+} stores by thapsigargin. AII, bradykinin, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) also caused a rise in $[Ca^{2+}]_i$ in a subset of the cells. ATP, basic fibroblastic growth factor (bFGF), EGF and VEGF induced mitogenesis and caused a rise in ERK 2 activation within 10 min. L-Arginine to L-citrulline conversion assays showed that ATP, EGF and VEGF induced a significant rise in eNOS activity, and this correlates with an ability to induce Ca^{2+} mobilization and ERK 2 activation. In conclusion, HUVEC-CS are indeed endothelial cells and appear to be functionally very similar to primary HUVEC. These cells will prove a valuable tool for future studies in both basic and therapeutic sciences.

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Introduction

Endothelial dysfunction has been linked to many human illnesses, ranging from obesity and diabetes to pre-eclampsia. Therefore, reliable endothelial cell lines have been sought for many years. In addition to providing a uniform model to study endothelial cell function, they also have the advantage of providing a starting point for the generation of subsequent sublines selected by function or altered by transformation. Cell lines of human origin are particularly valuable because there is limited access to human tissue. Where available, primary cultures of endothelial cells have been used to study endothelial cell physiology, but they often show large degrees of heterogeneity between preparations. Differences between individuals as well as in culture conditions can contribute to such variations.

In view of these difficulties, it is not surprising that endothelial cell lines are immediately put to use as soon as they are described in the literature. However, the number of cell lines available is limited. It has been recently reported that endothelial cells from different tissues are locally specialized in their functional characteristics. The cells express specific subsets of genes according to tissue of origin as well as source (arteries versus veins or macrovasculature versus microvasculature) (Chi *et al.* 2003). Thus, the few lines that are available are not sufficient to model endothelial cell function in multiple vascular beds; rather, a stable cell line is needed for each vascular bed.

Most of the endothelial cell lines that are currently in use have been described as being derived from primary human umbilical vein endothelial cells (HUVEC). HUVEC-C is one such cell line; it is an immortalized human umbilical vein endothelial cell line that is commercially available from ATCC (American Type Culture Collection). Like many of its predecessors, it is potentially a useful tool to study endothelial cell function, yet little research has been done to characterize these cells. Many

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laboratories have begun to use these cells to study endothelial cell function without first confirming that these cells maintain endothelial cell characteristics. The dangers of failing to characterize the cell lines have been amply demonstrated in the past with ECV304 cells. ECV304 cells were also described as a spontaneously transformed line derived from human umbilical vein endothelial cells (Takahashi *et al.* 1990). However, about 10 years after the cells became available, the ECV304 cell line was characterized, and it became apparent that these cells had both endothelial and epithelial features (Kiessling *et al.* 1999, Brown *et al.* 2000, Suda *et al.* 2001). After DNA karyotyping by several laboratories, which was confirmed by ATCC, ECACC (European Collection of Animal Cell Cultures), DSMZ (German Collection of Microorganisms and Cell Cultures) and JCRB (Japanese Collection of Research Biosources), it became apparent that ECV304 cells are actually identical to T24/83 cells, which are a human bladder cancer cell line (Lidington & Moyes 1999, MacLeod *et al.* 1999, Tanabe *et al.* 1999, Unger *et al.* 2002). Despite this, ECV304 cells are still widely reported as a model for endothelial cell function.

Ea.hy926 is another widely used endothelial cell line. These cells were developed as a fused line between HUVEC and human pulmonary adenocarcinoma A549 cells (Edgell *et al.* 1983). This cell line has also been extensively characterized. Unger *et al.* found that, phenotypically, the Ea.hy926 cells closely resemble primary HUVEC. However, one of the main problems with Ea.hy926 cells is that they are not commercially available.

Besides being cross-contaminated or not commercially available, many primary as well as endothelial cell lines have been characteristically hard to maintain. Researchers report problems with long doubling times and poor substrate attachment. This problem is often solved by adding endothelial growth supplements to the media and/or growing the cells on Matrigel. While this can improve culture, it often complicates experimentation because it introduces growth factors. Thus, if the aim of the research is to study the effects of growth factors on endothelial cell function, the experiment cannot be performed. Stable cell lines grown under standard conditions would allow many laboratories to study endothelial cell function and easily compare data. Therefore, the aim of this study is threefold: 1) to develop a subline of the HUVEC-C which grow well in standard media without the introduction of supplemented growth factors, 2) phenotypically to determine whether the HUVEC-CS subline maintains endothelial cell characteristics in culture (as the ATCC has already karyotyped these cells), and 3) to functionally characterize the subline in its responsiveness to agonists. If these cells prove to be a valid endothelial cell line, they will be very valuable because not only are they widely available but also, because they are of human origin, they can be used with human proteomics and genomic tools/analyses. Likewise, these cells could be used as a clinical diagnostic tool. Once 'normal' is described in these cells, they could be compared with primary HUVEC cells derived from diseased pregnancies to help elucidate the underlying cause of endothelial dysfunction.

Materials and Methods

Materials

Primary and secondary antibodies were purchased from the companies listed in Table 1. MEM D-Val and all other cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). ATP (disodium salt), insulin (sodium salt), angiotensin II (AII) and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Growth factors (basic fibroblastic growth factor (bFGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) were purchased from R & D Systems (Minneapolis, MN, USA). Gelatin-coated T75 flasks (Biocoat, Beckton Dickinson Labware, Bedford, MA, USA), tissue-culture treated T75 flasks and eight-well glass chamber slides (Nunc, Fisher Scientific) were distributed by Fisher Scientific (Itasca, IL, USA). Glass bottom microwell dishes for Ca^{2+} imaging studies were from MatTek Corporation (Ashland, MA, USA).

Cell culture

HUVEC-C were purchased from ATCC Manassas, VA, USA. Cells were initially plated to raw plastic, tissue culture-treated T75 flasks and grown for six passages (1:2 split) in DMEM containing 10% fetal bovine serum (FBS) (cat. no.16000–036), 10% fetal calf serum, 1 unit/ml penicillin, 1 μg/ml streptomycin and 4 μg/ml gentamicin (Sigma). They were trypsinized and transferred to medium containing 10% dimethyl sulfoxide (DMSO), frozen in liquid nitrogen and designated HUVEC-CS. Cells were then plated to gelatin-coated T75 flasks and grown to approximately 70% confluence for five more passages in MEM D-Val (Cat. no. 11095080) containing 20% FBS, 1 unit/ml penicillin, 1 μg/ml streptomycin and 4 μg/ml gentamicin. They were then passaged to medium containing 10% DMSO and frozen in liquid nitrogen for long-term storage.

Acetylated low-density lipoprotein (AcLDL) uptake

HUVEC-CS passage 6 were seeded at various densities and cultured to 25–75% confluence on eight-well chamber slides. Cells were then incubated for 4 h at 37 °C in 200 μl MEM containing 20% FBS, 1 unit/ml penicillin, 1 μg/ml streptomycin and 4 μg/ml gentamicin with or without 10 μg/ml AcLDL (Biomedical Technologies,

Table 1 Expression of proteins in HUVEC-CS

Protein	Positive (+) or negative (−)	Detection method(s)	MW	1st antibody (company, catalog no., dilution)	2nd antibody (type‡ dilution)
Proteins associated with nitric oxide production					
iNOS	−	Western	130	Transduction Labs, N32020, 1:500	C, 1:2500
nNOS	−	Western	155	Upstate, 06-528, 1:1000	A, 1:2000
eNOS	++++	Western	140	Transduction Labs, N30020, 1:750	C, 1:2500
Cav-1	++++	Western	20	Santa Cruz, sc-894, 1:40 000	A, 1:10 000
Cav-2	++++++	Western	20	Transduction Labs, C57820, 1:500	C, 1:2500
Cav-3	−	Western	18	Transduction Labs, C38320, 1:1000	C, 1:2500
HSP90	++++	Western	90	Affinity Bioreagents, PA3-013, 1:20 000	A, 1:10 000
Proteins associated with prostacyclin production					
COX-1	+	Western	69	Cayman Chemicals, 160110, 1:5000	C, 1:5000
COX-2	++	Western	70	Cayman Chemicals, 160107, 1:2000	A, 1:2000
$cPLA_2$	+	Western	110	Santa Cruz, sc-454, 1:200	C, 1:2500
PGIS	−	Western	50	Cayman Chemicals, 160640, 1:30 000	A, 1:2500
Kinases					
Akt	++++	Western	55–60	New England Biolabs, 9272, 1:1000	B, 1:2000
ERK 1/2	++++	Western	42/44	New England Biolabs, 9102, 1:1000	B, 1:2000
G-Proteins					
Gαi-1	−	Western	42	Calbiochem, 371720, 1:2000	A, 1:2000
Gαi-2	++	Western	40	Calbiochem, 371723, 1:1000 (Antibody for Gαi-1/2)	A, 1:2000
Gαi-3	++	Western	45	Calbiochem, 371729, 1:2000	A, 1:2000
Gαi-o	++	Western	42	Calbiochem, 371726, 1:2000 (Antibody for Gαi-3/o) Santa Cruz, sc-387, 1:200	A, 1:2000 A, 1:2000
Gαq	+	Western	42	Calbiochem, 371752, 1:2000 Santa Cruz, sc-393, 1:200	A, 1:2000 A, 1:2000
Gαz	+	Western	41	Santa Cruz, sc-388, 1:200	A, 1:2000
Gαs	−	Western	45	Calbiochem, 371732, 1:1000	A, 1:2000
Gβ	+	Western	35, 36	Calbiochem, 371838, 1:2000	A, 1:2000
Purinoceptors					
P2X1	+	Western+BP	58	Alomone Labs, APR-001, 1:500	A, 1:2000
P2X2	+	Western+BP	200	Alomone Labs, APR-003, 1:500	A, 1:2000
P2X4	+	Western	55	Alomone Labs, APR-002, 1:500	A, 1:2000
P2X7	+	Western+BP	75/80 (doublet)	Alomone Labs, APR-004, 1:500	A, 1:2000
P2Y1	−	Western+BP	220	Alomone Labs, APR-009, 1:500	A, 1:2000
P2Y2	+++	Western+BP	53	Alomone Labs, APR-010, 1:500	A, 1:2000
P2Y4	−	Western	81	Alomone Labs, APR-006, 1:500	A, 1:2000
Growth factor receptors					
KDR	+	Western	180–210	Santa Cruz, sc-505, 1:500 (Western)	A, 1:2000
	++++	ICC		Santa Cruz, sc-505, 2 µg/ml (ICC)	E, 1:200
EGFR	++	ICC	180	Transduction Labs, 610016, 2 µg/ml	D, 1:200
Flg-1	+++	ICC+BP	120	Santa Cruz, sc-121, 1 µg/ml	E, 1:200
Flt-1	+++	ICC+BP	180	Santa Cruz, sc-316, 1 µg/ml	E, 1:200
Heptahelical receptors					
B2R	+++	Western	42	Transduction Labs, B40820, 1:2000	C, 1:2500
AT_1R	+	Western	54	Santa Cruz, sc-579, 1:750 (Western)	A, 1:2500
	+++++	ICC		Santa Cruz, sc-579, 2 µg/ml (ICC)	E, 1:200
AT_2R	+++ (31) ++ (46 & 65)	Western	31, 46, 65	*Made by IM Bird, 1:2000	A, 1:2000
Other proteins					
ZO-1	++++	Western	225	Zymed Laboratories, 61-7300, 1:200	A, 1:2500
IP3R3	++++	Western	240	Transduction Labs, 131220, 1:1000	C, 1:2500

Proteins were detected in HUVEC-CS by using the method indicated, as described in the Methods section. BP indicates that a blocking peptide was used in conjunction with ICC or Western blot analysis to show further specificity. Proteins are indicated as positive (+) or negative (−). Stronger bands on western blots or more intense staining for ICC are indicated by more +, with +++++ being the best detected. ‡Secondary antibodies listed in the table are as follows: A) donkey antirabbit (Amersham, Piscataway, NJ, USA, cat. no. NA9340); B) donkey antirabbit (Cell Signaling Technologies, Beverly, MA, USA, cat. no. 7074); C) Sheep antimouse (Amersham, cat. no. NA9310); D) horse antimouse (Vector Labs, Burlingame, CA, USA, cat no. 6102); E) goat antirabbit (Vector Labs, cat. no. 6101). iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; $CPLA_2$, cytosolic phospholipase A_2.

Stoughton, MA, USA). The slides were then rinsed with PBS and mounted, using mounting medium with DAPI (Vector Labs, Burlingame, CA, USA; cat no. H-1200) to stain the nuclei. The slides were then imaged with a fluorescent microscope.

Immunocytochemistry (ICC)

HUVEC-CS passage 6 (for endothelial nitric oxide synthase (eNOS), Ki67, proliferating cell nuclear antigen (PCNA), and phospho-specific (P-ERK) 1/2) or HUVEC-CS passage 0 (ve-cadherin and CD31) (platelet endothelial cell adhesion molecule (PECAM-1)) were cultured to a subconfluent state in eight-well chamber slides. Cells that would subsequently be immunostained for P-ERK 1/2 were serum starved for 4 h before stimulation with agonist for 10 min at the appropriate doses (AII 100 nM, ATP 100 μM, bFGF 10 ng/ml, EGF 10 ng/ml and VEGF 10 ng/ml). In some of the P-ERK 1/2 experiments, half of the chambers were pretreated with 10 μM U0126 for 20 min. Following stimulation, cells were rinsed with ice-cold PBS and fixed for 1 h at room temperature with 4% paraformaldehyde in 0·1 M sodium cacodylate buffer, pH 7·5 (Electron Microscopy Sciences, Fort Washington, PA, USA). In probing for eNOS, Ki67, PCNA or P-ERK 1/2, before blocking, the cells were permeabilized with 0·3% Triton X-100 to ensure the antibody could penetrate the nucleus. Cells were incubated with the appropriate primary antibody for 2 h at room temperature (CD31, 4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA; ve-cadherin, 4 μg/ml, Santa Cruz Biotechnology; eNOS, 0·5 μg/ml, Transduction Laboratories (BD Biosciences, San Diego, CA, USA); phospho-specific ERK 1/2 (P-ERK 1/2), 0·668 μg/ml, Promega; PCNA, 0·125 μg/ml, Oncogene Research Products, Boston, MA, USA; Ki67, 1·0 μg/ml, Novocastra Laboratories, Newcastle, UK). After rinsing with PBS, the cells were incubated in the secondary antibody according to the manufacturer's specifications (Vector Laboratories). Localization of the protein was visualized by indirect immunoperoxidase detection via the avidin-biotinylated peroxidase complex method with 3,3′-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) according to the manufacturer's directions (Vector Laboratories). Controls consisted of preimmune rabbit or mouse IgG (Vector Laboratories) at the same concentration as the primary antibody in place of the antibody. After immunostaining, the cells were counterstained with Harris modified hematoxylin with acetic acid (Fisher Scientific) for 7 s to visualize unstained cells.

Capillary tube formation

HUVEC-CS passage 0 were cultured to confluence in a tissue culture-treated T75 flask. At 24 h prior to the experiment, the growth media (DMEM containing 20% FBS, 1 unit/ml penicillin, 1 μg/ml streptomycin and 4 μg/ml gentamicin) was replaced with serum-free media. After 24 h of serum deprivation, the cells were trypsinized, seeded at 20 000 cells/well to 96-well Optilux Plates (BD Biosciences) coated with 100 μl of growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA, USA; cat no. 356237) and incubated for 8 h at 37 °C. The media was removed, and the cells were fixed and stained with 4% formalin (Electron Microscopy Sciences) containing 0·01% crystal violet for 30 min at room temperature. The cells were rinsed twice with dH_2O and then imaged on a Nikon TE2000U microscope.

Total cellular protein lysate isolation and Western blot analysis

At 70% confluency, HUVEC-CS passage 6 were rinsed twice with ice-cold PBS and lysed in 500 μl phosphoprotein lysis buffer (4 mM sodium pyrophosphate, 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM $NaVO_4$ with 1 mM PMSF, 1·0% Triton X-100, 5 μg/ml leupeptin and 5 μg/ml aprotinin). Sonicated lysates were centrifuged at 10 000 ***g*** for 10 min to pellet cellular debris. Protein concentration was determined by the BCA assay. An amount of 150 μg protein was loaded across the entire width of a 1 mm, 7·5% or 10% gel (cat. no. 165–2928; BioRad, Hercules, CA, USA). The lysate and 'rainbow' molecular weight marker (Amersham; Piscataway, NJ, USA) were separated by SDS–PAGE (2 h, 100 V) and electroblotted (2 h, 100 V) to an Immobilon-P membrane (Millipore, Bedford, MA, USA) with the Mini Protean II electrophoresis system (BioRad). Blots were blocked in 0·5% fat-free milk as previously described (Zheng 1998) and probed with primary antibodies (2 h, room temperature) and HRP-conjugated secondary antibodies (1 h, room temperature) before detection of signal with the enhanced chemiluminescence (ECL) reagent system and HyperFilm (Amersham). Primary and secondary antibody dilutions and sources are listed in Table 1.

Intracellular Ca^{2+} detection

HUVEC-CS passage 6 cells were grown in 35 mm, glass-bottom microwell dishes for 1–7 days to allow attachment before experimentation. Cells were then incubated in Krebs buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 6 mM glucose, 25 mM HEPES and 2 mM $CaCl_2$ (Calbiochem; San Diego, CA, USA), pH 7·4) and loaded with 5 μM Fura-2 AM (Molecular Probes, Inc.; Eugene, OR, USA) in the presence of 0·05% pluronic F127 (Molecular Probes) for 45 min at 37 °C. The cells were then washed twice, covered in 1 ml Krebs buffer and incubated for a further 30 min to allow complete ester hydrolysis. Cells were washed again and covered with 1 ml Krebs buffer. Fura-2 loading was verified by viewing at 380 nm UV excitation on a Nikon inverted microscope. A single, isolated cell was

Table 2 Calcium imaging and multiagonist stimulation

	Single cells with ↑ $[Ca^{2+}]_i$	Total no. of single cells tested	% of single cells with ↑ $[Ca^{2+}]_i$	Groups with ↑ $[Ca^{2+}]_i$/ groups tested	No. of cells in all groups	% of cells with ↑ $[Ca^{2+}]_i$
Agonist						
AII	2	14	14·29	3/7	58	42·86
BK	7	15	46·67	4/7	37	57·14
bFGF	0	5	0·00	0/5	31	0·00
EGF	0	6	0·00	3/5	31	60·00
VEGF	1	6	16·67	2/5	31	40·00

Agonists other than ATP were tested to determine whether they could initiate a rise in intracellular free calcium ($[Ca^{2+}]_i$). When single cells were isolated and stimulated with 100 nM AII, 1 μm bradykinin (BK), 10 ng/ml bFGF, 10 ng/ml EGF or 10 ng/ml VEGF, it was found that a subset of the cells responded to AII, BK and VEGF, but none of the cells responded to bFGF or EGF. To determine that the low percentage of responding cells was due to the level of confluency, the experiment was repeated with groups of cells that were touching. The results indicate that in HUVEC-CS, the rise in $[Ca^{2+}]_i$ after AII, EGF or VEGF stimulation is largely increased by increased confluency, while the response to BK is only slightly improved. It also appears that HUVEC-CS do not respond to bFGF by initiating a rise in $[Ca^{2+}]_i$.

placed in the field of view, and 10-min recordings commenced with alternate excitation at 340 and 380 nm at 50-ms intervals, and measuring emitted light at 510 nm by a photomultiplier. From the ratio of emission detected at the two excitation wavelengths and by comparison to a standard curve established for the same settings using buffers of known free $[Ca^{2+}]$, the $[Ca^{2+}]_i$ was calculated in real time by InCyt Pm2 software (InCyt, Cincinnati, OH, USA) (Bird *et al.* 2000, Di *et al.* 2001). An ATP dose response was established by adding 1, 3, 10, 30, 100 and 300 μM ATP in random order with 20-min recovery periods between treatments ($n=5$ for 3–300 μM; $n=3$ for 1 μM). To determine the pathway of Ca^{2+} release, the cells were treated with thapsigargin (10 μM, Calbiochem), and once the $[Ca^{2+}]_i$ had returned to the basal level, 30 μM ATP was added ($n=8$). To determine the role of L-type channels in the Ca^{2+} response, cells were treated with ATP, allowed to recover for 20 min and then pretreated with either verapamil (1 and 10 μM, Calbiochem) or nifedipine (1 and 10 μM, Calbiochem) for 5 min before stimulation with 30 μM ATP. After recovering for 20 min, the cells were stimulated with 30 μM ATP once again to compare with the initial ATP treatment ($n=7$ for verapamil; $n=6$ for nifedipine). BAYK8644 experiments involved treating the cells with 30 μM ATP, a 20-min recovery, stimulation with BAYK8644 (1 or 10 μM, Calbiochem) and recovery, followed by treatment with 30 μM ATP ($n=4$). To determine whether other agonists could induce Ca^{2+} mobilization and whether cell density affected this response, individual cells or groups of 2–20 cells were treated with ATP (30 μM) to ensure the cells were viable. Then they were treated with AII (100 nM), bFGF (10 ng/ml), EGF (10 ng/ml), VEGF (10 ng/ml) and/or bradykinin (BK, 1 μM) in random order with 20-min recovery periods between treatments. Not every cell or group of cells was stimulated with every agonist. See Table 2 for *n* values.

P-ERK 2 assay

HUVEC-CS passage 6 were trypsinized from a gelatin-coated T75 flask to seven 35 mm dishes. After incubation overnight, cells underwent serum withdrawal for 1 h in 1·8 ml MEM containing 0·1% BSA, 1 unit/ml penicillin, 1 μg/ml streptomycin and 4 μg/ml gentamicin. Cells were stimulated with 200 μl agonists for 10 min at the appropriate doses (as above). The reactions were terminated by addition of 2 ml ice-cold PBS. Cell lysates were prepared as described above in the 'Total cellular protein lysate isolation and Western blot analysis' section except that 50 μl lysis buffer was used in the 35 mm dishes. An amount of 10 μg protein was loaded into each lane of a 28-well, 1-mm-thick, 10% gel by the Criterion electrophoresis system (Biorad). The lysate and 'rainbow' molecular weight marker (Amersham) were separated by SDS–PAGE (1 h, 200 V) and electroblotting (1 h, 100 V) to an Immobilon-P membrane (Millipore). Membranes were blocked in 0·5% fat-free milk, probed with the primary antibody for 2 h, rinsed and incubated with the secondary antibody for 1 h at room temperature. The membranes were washed again, and the signal was detected with the ECL reagent system and HyperFilm (Amersham). Each membrane was first probed with anti-phospho p42/p44 MAPK pAb (Promega) (1:5000) and donkey-antirabbit secondary antibody (Cell Signaling Technology) (1:2000) for 1 h at room temperature. After freezing of the membrane to inactivate the HRP conjugate, membranes were blocked and then probed for total p42/p44 MAPK pAb (New England Biolabs, Beverly, MA, USA) (1:1000), using donkey antirabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) (1:2000). After another round of freezing and blocking, each membrane was probed for phospho-specific Akt (Cell Signaling Technology) (1:750) with donkey antirabbit HRP-conjugated secondary antibody (Cell Signaling Technology) (1:7500). Films were scanned for

densitometry, and the data were normalized to total ERK 1/2 for that lane and expressed as fold of control.

eNOS activation assay

HUVEC-CS passage 6 were plated in 35 mm dishes and allowed to attach overnight. Prior to stimulation, cells were washed twice with 2 ml prewarmed Krebs and incubated for 1 h in 900 µl Krebs buffer. An amount of 3 µCi ^{3}H-arginine monohydrochloride (61 Ci/mmol in 50 µl) (Amersham) was added to each dish, followed immediately by addition of 50 µl agonist (as above), and the cells were incubated for 30 min. Reactions were stopped with 400 µl ice-cold 15% perchloric acid (final concentration 5%). An amount of 100 nCi ^{14}C-arginine monohydrochloride (310 mCi/mmol in 100 µl) (Amersham) was added to each dish for data normalization. After scraping, all of the lysate (1·5 ml) was collected, and the dish was rinsed with 1 ml nanopure water, which was added to the lysate. Insoluble cell debris was removed with 5 min of centrifugation at 12 000 ***g***. Samples were extracted, using 1·5 ml of a 1:1 mix of 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine. Then 900 µl of the extracted samples were applied to AG 50W-X8 cation-exchange resin (Biorad), and citrulline flow through was collected, using a total of 7·5 ml of 25 mM HEPES, pH 5·5, 2 mM EDTA and 2 mM EGTA. Arginine was eluted from the column with a total of 8 ml of 50 mM KOH into a separate scintillation vial. InstaGel scintillation fluid (Packard Biosciences, Meridan, CT, USA) was added to each wash, and the samples were counted on the TRI-CARB 2300TR liquid scintillation analyzer (Packard Biosciences). A ratio of ^{3}H citrulline counts to total ^{14}C was calculated for each sample, the blank ratio was subtracted from this ratio, and then the data were expressed as fold of ratio for the unstimulated control.

Mitogenesis assay

HUVEC-CS passage 6 were grown in a gelatin-coated T75 flask to 70% confluency and then passaged to two 12-well plates. The cells were allowed to attach overnight before beginning the 4-h serum starvation in 900 µl MEM containing 0·1% BSA, 1 unit/ml penicillin, 1 µg/ml streptomycin, and 4 µg/ml gentamicin. The cells were stimulated to a total volume of 1 ml with vehicle, ATP (100 µM), AII (100 nM), insulin (1 µM), bFGF (10 ng/ml), EGF (10 ng/ml) or VEGF (10 ng/ml) for 12 h in serum-free media. An amount of 1 µCi ^{3}H-methyl thymidine (Amersham) was then added to each well and allowed to incorporate for a further 4 h. The reaction was stopped with ice-cold PBS. The cells were then washed in 5% perchloric acid to remove unincorporated labeled precursors to DNA. After rinsing of the cells, they were solubilized in 1% Triton X-100/0·1% NaOH. The solubilized cells were transferred to scintillation vials with 5 ml of scintillation cocktail (Packard Biosciences, cat. no. 6013399). The data was collected using a TRI-CARB 2300TR liquid scintillation analyzer (Packard Biosciences) counting for 5 min or to 1% error.

Statistics

All protein analyses and cellular function experiments were repeated at least three different times or as stated. Each treatment in Ca^{2+} imaging of Fura 2 loaded cells was replicated at least three times. Student's *t*-test or one-way ANOVA with Bonferroni *t*-test was used to analyze the data as appropriate. $P<0·05$ was considered significant.

Results

Selection and maintenance of endothelial cell characteristics

ATCC grows and recommends growing HUVEC-C (the original cell line) in Ham's F12K medium supplemented with endothelial cell growth supplement among other things. Therefore, our first goal was to select an adherent subpopulation by growing the cells on tissue culture-treated, uncoated T75 flasks in more standardized media without supplements. Thus, the cells were grown in DMEM on raw plastic for six passages. Cells floating in the media either because they did not adhere or had died in the media were discarded between passages.

ATCC has already performed DNA karyotyping on the HUVEC-C cells; therefore, we did not repeat this. However, due to the selection process, we did want to verify that the subline, HUVEC-CS, maintained endothelial cell characteristics and were healthy and proliferating. To achieve this aim, several well-established markers for endothelial cells were evaluated. All of the cells took up AcLDL and expressed eNOS, and the majority of cells expressed CD31 and ve-cadherin (Fig. 1). In addition, when these cells were grown on Matrigel, they spontaneously formed capillary tube-like structures (Fig. 1H). These data confirm that the cells retain many functional characteristics of endothelial cells.

Next, the cells were stained with two markers of cell-cycle progression. Ki67 (a nuclear protein, which is expressed in G1, S, G2 and M phases of the cell cycle, but not in G0) has a half-life of approximately 4 h. Figure 2A shows cells that were immunostained with Ki67 and thus that a portion of the cells had gone through the cell cycle 4 h prior to being fixed. All of the cells in Fig. 2B show positive immunostaining for PCNA, which is mainly expressed during the S phase. PCNA has a half-life of about 24 h; therefore, all of the HUVEC-CS had divided sometime within the day before fixation. Thus, these cells are healthy and proliferating.

Protein expression

After verification that the subline HUVEC-CS maintained endothelial cell characteristics, Western blot analysis

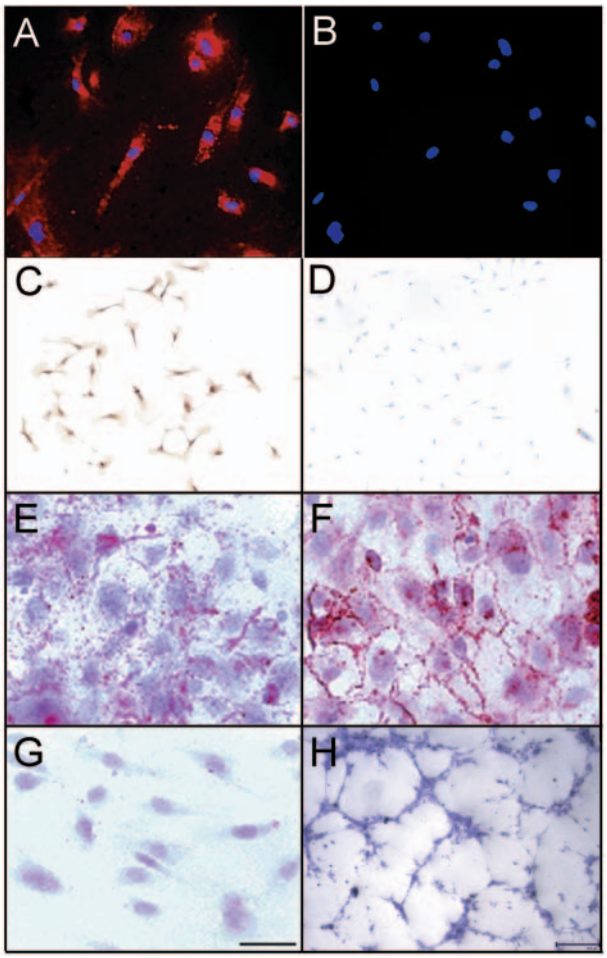

Figure 1 Endothelial cell markers in HUVEC-CS. Cells were labeled/stained as described in Methods. (A) AcLDL uptake shown in red with DAPI-stained nuclei, (B) control with DAPI only, (C) eNOS immunolocalization shown in brown and counterstained with hematoxylin, (D) control IgG for panel C, (E) CD31 immunolocalization shown in red and counterstained with hematoxylin, (F) ve-cadherin immunolocalization shown in red and counterstained with hematoxylin, (G) IgG control for panels E and F (bar=40 μm), (H) tube formation on Matrigel (bar=100 μm).

and ICC were utilized to determine whether these cells expressed many of the key proteins necessary for agonist-induced vasodilator production. Table 1 summarizes the results. Briefly, HUVEC-CS expressed eNOS, but not iNOS or nNOS. They also expressed HSP90, caveolin-1 and caveolin-2, but not caveolin-3. Likewise, COX-1, COX-2 and cPLA2 were expressed, but PGIS was not detectable. HUVEC-CS also expressed many purinergic receptors. A variety of ligand-gated ion channels were detected, including P_2X_1, P_2X_2, P_2X_4 and P_2X_7; however, of the G-protein-coupled receptors that were evaluated, only P_2Y_2 was detected. Receptors for VEGF, EGF, bFGF, bradykinin (B2R) and AII (AT_1R and AT_2R)

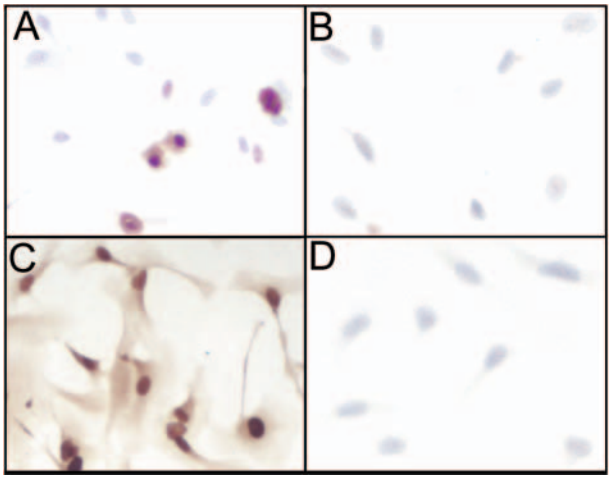

Figure 2 Markers of cell proliferation. (A) Cells immunostained for Ki67. (B) Control IgG at the same concentration as Ki67 gave no nuclear staining. (C) Positive nuclear staining was seen in every cell immunostained for PCNA. (D) No nuclear staining was seen in the IgG control. These data demonstrate that the HUVEC-CS are healthy and proliferating.

were also present. HUVEC-CS also appeared to express many of the proteins required for downstream signaling, including kinases (Akt and ERK 1/2) as well as several G-protein subunits (Gαi-2, Gαi-3, Gαi-o, Gαq, Gαz and Gβ, but not Gs). HUVEC-CS were also shown to express ZO-1, which is a protein involved in the formation of tight junctions.

Ca^{2+} imaging

The ability of HUVEC-CS to mobilize Ca^{2+} in response to ATP was evaluated. The general Ca^{2+} response induced by 30 μM ATP was biphasic with an initial peak in the $[Ca^{2+}]_i$ followed by a prolonged sustained phase. First, a dose-response curve was established for the ATP-induced Ca^{2+} response. The rate of initial response to ATP was highly variable; some cells responded to the ATP instantaneously while others were delayed for up to 9 min. Therefore, both the peak response and the delay of the response were used to determine the optimal dose of ATP. A dose of 30 μM ATP was chosen as optimal because it caused the largest peak response and because the delay of the response was significantly lower at this dose (Fig. 3).

To determine whether ATP caused a release of Ca^{2+} from intracellular stores in HUVEC-CS, the cells were treated with 10 μM thapsigargin followed by ATP treatment. Thapsigargin inhibits the Ca^{2+} ATPase on the endoplasmic reticulum (ER) and leads to the depletion of the $[Ca^{2+}]$ in the ER. ATP was unable to initiate a response after the ER Ca^{2+} stores had been emptied (Fig. 4), implying that the ATP receptor involved in producing the initial transient peak does not cause

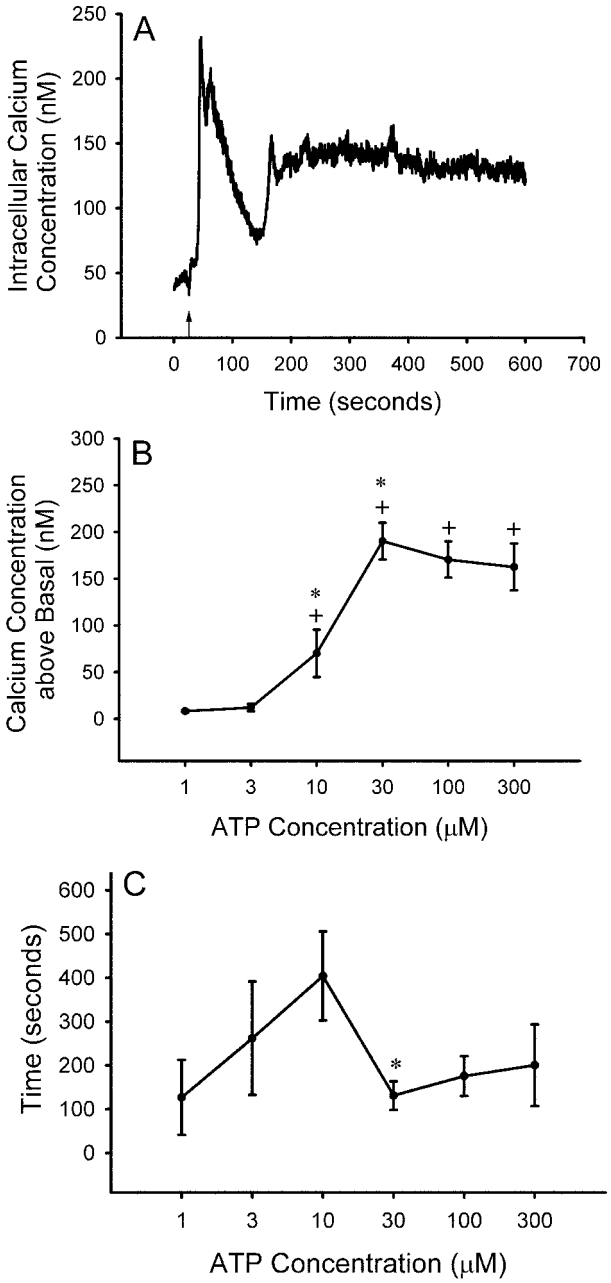

Figure 3 ATP dose response in HUVEC-CS. Cells were plated and allowed to attach overnight. The cells were loaded with Fura 2 and imaged. The basal $[Ca^{2+}]_i$ was recorded for 30 s before stimulation with various doses of ATP. (A) Representative graph of the change in $[Ca^{2+}]_i$ in response to 30 μM ATP. The arrow marks the time point at which 30 μM ATP was added. (B) Graph of the maximal $[Ca^{2+}]_i$ above basal achieved after stimulation with various concentrations of ATP. (C) Graph of the time it took to reach the maximum after stimulation with ATP. $n=5$ for all doses except 1 μM ($n=3$). Mean ± S.E. $^{+}P \leq 0{\cdot}05$ as compared with basal $[Ca^{2+}]_i$, $^{*}P \leq 0{\cdot}05$ as compared with immediate lower dose of ATP.

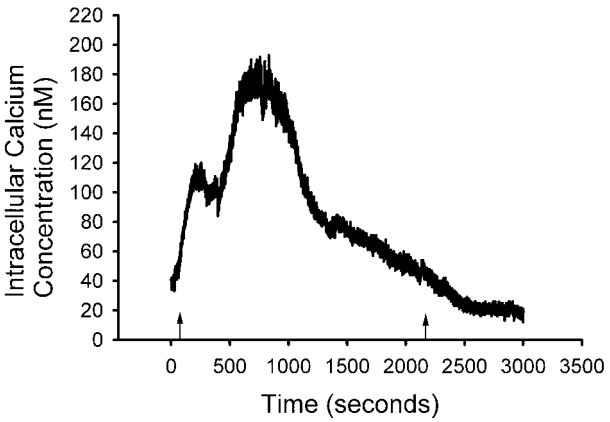

Figure 4 The ATP receptor works through internal calcium stores. Cells were plated, allowed to attach overnight and then loaded with Fura 2. This is a representative graph of the change in $[Ca^{2+}]_i$ in response to 30 μM ATP (marked by the second arrow) after the intracellular pools of Ca^{2+} had been depleted by treatment with 10 μM thapsigargin (marked by first arrow). The inability of ATP to initiate a rise in the $[Ca^{2+}]_i$ after the intracellular stores had been depleted shows that ATP works to increase the $[Ca^{2+}]_i$ by causing Ca^{2+} release from the endoplasmic reticulum. Similar responses were seen in eight different experiments on eight different cells.

extracellular Ca^{2+} influx but rather causes the release of Ca^{2+} from the intracellular stores.

As noted above, the Ca^{2+} response to ATP was biphasic. Typically, the initial peak is caused by the release of Ca^{2+} from the intracellular stores, but the sustained phase is due to the influx of Ca^{2+} from the extracellular space during capacitative Ca^{2+} entry. The influx of Ca^{2+} from the extracellular space can be caused by a wide variety of channels, including L-type channels. To determine whether L-type channels were involved in the ATP-induced response, verapamil, an L-type channel inhibitor, was used in conjunction with ATP. Verapamil caused a significant decrease in the ATP-induced response; however, it was not dose-dependent. The mean response after inhibition by 10 μM verapamil was higher than inhibition by 1 μM verapamil ($n=7$; data not shown). To rectify the ambiguous results, the same experiment was repeated with another L-type channel inhibitor, nifedipine. This time, the response to ATP was not significantly different when treated alone or pretreated with 1 or 10 μM nifedipine ($n=6$; data not shown). For further verification that L-type channels were truly not involved in the ATP-induced Ca^{2+} response, one more set of experiments was performed, using an agonist for L-type channels, BAYK8644. BAYK8644 was unable to cause Ca^{2+} mobilization in these cells, while ATP did both at the beginning and end of each experiment ($n=4$; data not shown). Thus, L-type channels do not appear to be involved in the ATP-induced Ca^{2+} response in HUVEC-CS.

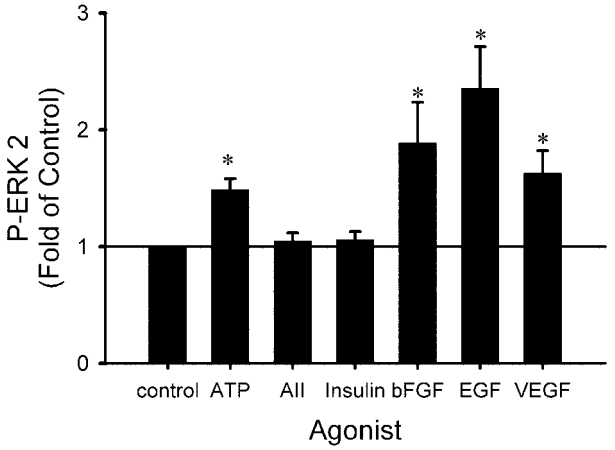

Figure 5 P-ERK 2 assay. Cells were stimulated with various agonists for 10 min and lysed, and the samples were run on SDS–PAGE. The proteins were transferred to a membrane, and the membrane was probed for active ERK 1/2 and total ERK 1/2. Densitometry was used to quantify the intensity of the ERK 2 bands, and the data were normalized to total ERK. ATP (100 μM), bFGF (10 ng/ml), EGF(10 ng/ml) and VEGF(10 ng/ml) could cause a significant rise in ERK 2 activation above the control value, but AII (100 nM) and insulin (1 μM) could not (mean ± S.E., $n=4$; $^*P \leq 0{\cdot}05$).

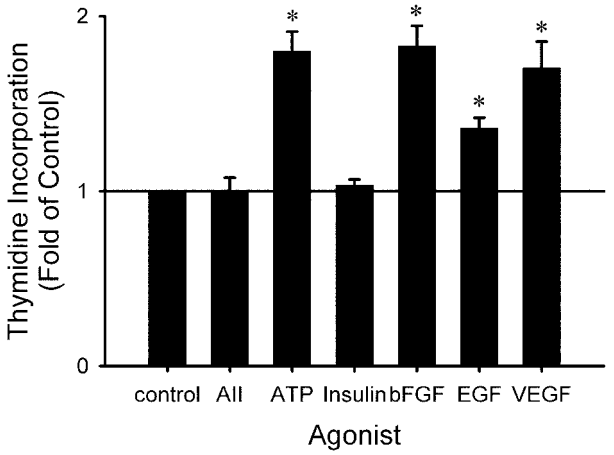

Figure 6 Thymidine incorporation. Cells were plated to 12-well dishes and allowed to attach overnight. After adhering, they underwent serum starvation for 4 h followed by agonist treatment (ATP 100 μM, AII 100 nM, insulin 1 μM, bFGF 10 ng/ml, EGF 10 ng/ml or VEGF 10 ng/ml) for 12 h. Next, thymidine was added to each well and allowed to incorporate for 4 h. The reaction was stopped with ice-cold PBS, and the cells were solubilized and then counted. ATP, bFGF, EGF and VEGF could all cause an increase in mitogenesis, but AII and insulin did not cause a change from the control level (mean ± S.E., $n=3$ in triplicate; $^*P \leq 0{\cdot}05$).

The ability of AII, BK, bFGF, EGF and VEGF to mobilize Ca^{2+} was assessed. A proportion of the individual cells responded to all of the agonists, except bFGF and EGF (Table 2). To analyze the effect of cell density on the ability of the previously tested agonists to induce Ca^{2+} mobilization, groups of touching cells (2–20 cells) were used. Increasing cell density resulted in more of the groups responding to all the agonists except bFGF (Table 2). However, these data were collected with a photomultiplier, which records the average response in the field of view; therefore, it is impossible to say whether more cells were responding to each agonist or whether some of the cells were just showing a response higher in magnitude. Yet, because the amplitude of the peak response was about the same for single cells as for groups of cells, it seems likely that more cells were responding at the same magnitude.

ERK 1/2 activation

Like Ca^{2+} signaling, ERK 1/2 activation is an important regulator of key endothelial cell functions, including mitogenesis and possibly vasodilator production at the level of cPLA2 and eNOS. Therefore, we analyzed the ability of the previously described agonists to induce ERK 2 phosphorylation in HUVEC-CS. Western blot analysis revealed that ATP, bFGF, EGF and VEGF all caused a significant rise in ERK 2 activation over the control, while AII and insulin did not (Fig. 5). These membranes were also probed for activated Akt, since this has been associated with activation of eNOS independently of Ca^{2+} mobilization. Of the agonists tested, only EGF caused acute phosphorylation of Akt (data not shown).

A similar set of experiments used ICC to determine whether more cells contained P-ERK 1/2 or whether the same amount of cells contained more P-ERK 1/2. ICC revealed that ATP, bFGF and EGF caused a larger population of cells to contain P-ERK 1/2 as compared with control. VEGF also caused a larger percentage of the cells to express P-ERK 1/2; however, this effect did not achieve significance (data not shown). The cells were simply scored as positive or negative, and the intensity of the stain was not scored in an attempt to lessen the subjectivity of the data analysis. Generally, the intensity of the staining was higher in the stimulated cells than control cells; therefore, it is likely that VEGF would have achieved significance if the intensity of the stain had also been scored. Therefore, we concluded that agonist stimulation caused phosphorylation in more cells and to a greater extent in those cells. Furthermore, no cell that had been exposed to the UO126 (a MEK inhibitor) showed immunostaining for activated P-ERK 1/2 (data not shown). This further validates the specificity of staining by the P-ERK 1/2 antibody.

Mitogenesis

The ability of ATP, AII, insulin, bFGF, EGF and VEGF to stimulate mitogenesis in HUVEC-CS was evaluated in a thymidine incorporation assay. Figure 6 showed that ATP, bFGF, EGF and VEGF were all able to cause a

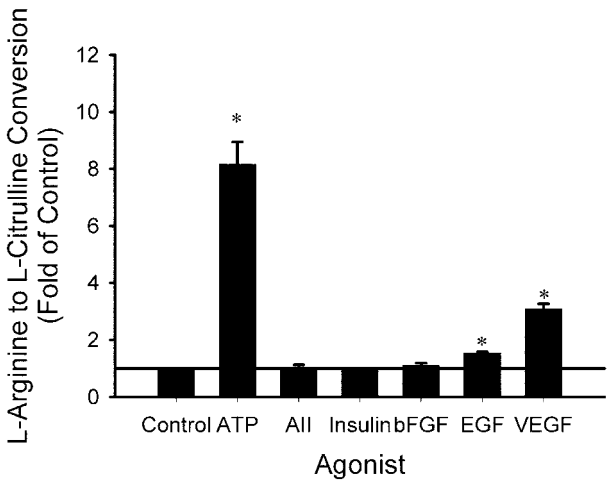

Figure 7 eNOS activity assay. Cells were plated to 35 mm dishes, allowed to attach overnight and then incubated in Krebs for 1 h. ^{3}H-Arginine and agonist (ATP 100 µM, AII 100 nM, insulin 1 µM, bFGF 10 ng/ml, EGF 10 ng/ml or VEGF 10 ng/ml) were added to each well and incubated for 30 min. The reaction was stopped with ice-cold PCA. ^{14}C-Arginine was added to each well and the sample collected. The sample was loaded onto a column, and the citrulline was eluded with HEPES and counted, and then the arginine was eluded with KOH and counted. The graph shows the fold of control. Neither insulin, AII nor bFGF caused a change in arginine to citrulline conversion. However, EGF, VEGF and ATP did cause an increase in citrulline accumulation (mean ± S.E., $n=4$ in duplicate; $^{*}P \leq 0{\cdot}05$).

significant increase in thymidine incorporation, while AII and insulin did not cause any change from the control level.

eNOS activity

L-Arginine to L-citrulline conversion assays were performed to determine whether any of the agonist tested could acutely activate eNOS in HUVEC-CS. Results show that neither insulin, AII nor bFGF caused detectable change in eNOS activity. However, EGF, VEGF and ATP all caused a significant increase in citrulline accumulation (Fig. 7).

Discussion

As noted above, it is extremely important to make sure that cell lines that have been reported to be endothelial cells do indeed maintain endothelial cell characteristics. We have shown that HUVEC-CS uniformly express eNOS, CD31 and ve-cadherin. These proteins have been considered classic markers of endothelial cells for many years. In addition, HUVEC-CS functionally maintain endothelial cell traits, including AcLDL uptake and tube formation on Matrigel. Furthermore, DNA karyotyping of the HUVEC-C (from which HUVEC-CS was derived) was performed by the ATCC, who found, both before and after karyotyping, no reason to believe that these cells were contaminated (personal communication). Together, these data strongly suggest that HUVEC-CS are a pure population of endothelial cells.

Once the subline had been selected and endothelial cell characteristics verified, it was important to confirm that all of the cells were actively going through the cell cycle if the line was to be of long-term use. To accomplish this aim, immunolocalization of Ki67 and PCNA was performed. Ki67 has been used for decades as a marker of progression through the cell cycle, as inhibition of Ki67 inhibits cell proliferation (Schluter *et al.* 1993). PCNA is involved in DNA replication in eukaryotes, and it, too, has been used as a marker for cell proliferation (Waseem & Lane 1990). In the cell, Ki67 has a half-life of 4 h while PCNA has a half-life of approximately 24 h. Thus, as expected, fewer cells showed immunostaining for Ki67 than for PCNA. Yet, all of the cells were positive for PCNA; therefore, all of the cells, not a subpopulation of them, are actively going through the cell cycle.

The production of vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2) by endothelial cells is essential in maintaining the balance between vasodilation and vasoconstriction (Schriffin 2001). Therefore, HUVEC-CS were evaluated to determine whether they expressed some of the key proteins necessary for vasodilator production. Western blot analysis showed that these cells express many of the proteins regulating NO production. For example, HUVEC-CS express eNOS, the enzyme that catalyzes the conversion of L-arginine to L-citrulline and NO. Many studies suggest that eNOS is post-translationally modified such that it is targeted on the caveolae. Being targeted on the caveolae plays a role in the regulation of eNOS because eNOS interacts directly with caveolin-1 (cav-1), the coat protein of the caveolae (Feron *et al.* 1996, García-Cardeña *et al.* 1996, Couet *et al.* 1997), in such a way that it covers the Ca^{2+}/calmodulin-binding domain. Since Ca^{2+}/calmodulin is necessary for eNOS activation, binding to cav-1 leaves eNOS in an inactive state (Ju *et al.* 1997, Michel *et al.* 1997*a*,*b*). These cells express cav-1. HSP90, a chaperone protein for eNOS, is also expressed. HSP90 binding, as well as Ca^{2+}/calmodulin, is associated with eNOS activation (García-Cardeña *et al.* 1998), and HSP90 has also been proposed to assist interaction of Akt with eNOS, as well as the interaction of Ca^{2+}/calmodulin with eNOS (Fontana *et al.* 2002, Takahashi & Mendelsohn 2003). The expression of these proteins confirms that HUVEC-CS are a valid cell line with which to study the molecular basis of eNOS activation.

Production of another potent vasodilator, PGI_2, involves several key steps. The action of cytosolic phospholipase A_2 ($cPLA_2$) on phospholipids to liberate arachadonic acid (AA) is the first and rate-limiting step in PGI_2 production. Only then can the COX-1 and/or COX-2 enzyme(s)

convert the liberated AA to PGG_2, which is subsequently converted to PGH_2. Finally, prostacyclin synthase (PGIS) converts PGH_2 to PGI_2 in many, but not all, endothelial cells. HUVEC-CS express $cPLA_2$, COX-1 and COX-2. However, they do not express detectable levels of PGIS. Therefore, while HUVEC-CS may be capable of prostanoid production in general, it is unlikely that HUVEC-CS would be capable of producing substantial amounts of PGI_2. This is consistent with the findings of Miralpeix *et al.* (1997) in the initial description of HUVEC-C. HPLC analysis of eicosanoids produced by HUVEC-C showed that the breakdown product of PGI_2, 6-keto-PGF1α, was produced in only minor amounts under resting or stimulated conditions. Thus, the lack of PGIS in HUVEC-CS does not appear to be a defect of the cell model, since while it is generally considered that PGI_2 is the main prostanoid produced by vascular endothelium, neither primary HUVEC nor porcine aortic endothelial cells produced much PGI_2 under unstimulated conditions (Bustos *et al.* 1997, Caughey *et al.* 2001). In other studies, endothelial cell stimulation has been reported to lead to the upregulation of COX-2, leading to increased PGI_2 production. Miralpeix *et al.* (1997) also noted an increase in COX-2 in association with increased prostanoid production, but they also noted that this was associated with an increase in prostanoids other than PGI_2.

One of the aims of this study was to evaluate several endpoints in order to determine how the HUVEC-CS functionally respond to a variety of agonists. Therefore, a combination of ICC and Western blot analysis was used to ascertain whether signaling molecules such as kinases, heterotrimeric G-proteins and receptors were expressed. While there are many cell-signaling kinases known to date, two, ERK 1/2 and Akt, are of particular relevance to this study because of their strong association with the regulation of mitogenesis and activation of eNOS and/or $cPLA_2$. Both Akt and ERK 1/2 were highly expressed in HUVEC-CS.

Likewise, heterotrimeric G-proteins are important for signal transduction for a variety of cellular functions. Gαq, and Gαi have been localized to the caveolae in smooth muscle cells (DeWeerd & Leeb-Lundberg 1997) and are the isoforms of the alpha subunit which are thought to couple to G-protein-coupled receptors to cause Ca^{2+} mobilization and subsequent eNOS activation (Boulanger & Vanhoutte 1997). Gαq and several isoforms of Gαi (Gαi-2, Gαi-3, Gαi-o, and Gαz but not Gαi-1) were expressed in HUVEC-CS. It is interesting to note that while many isoforms associated with Ca^{2+} and growth factor signaling were present, the isoform Gs was notably absent.

Western blot analysis also revealed that HUVEC-CS contain many purinergic receptors capable of binding ATP. Autocrine/paracrine signaling by way of ATP has proven to be important in several other endothelial cell types. ATP is able to induce Ca^{2+} mobilization and stimulate the activation of ERK 1/2 (Bird *et al.* 2000, Tran *et al.* 2000, Di *et al.* 2001, Kimura *et al.* 2001, Choi *et al.* 2002, Gifford *et al.* 2003). Since ATP is capable of stimulating so many diverse downstream effects, it is no surprise that there are many purinergic receptors. There are two main classes: namely, P_2X (ligand gated ion channels) and P_2Y receptors (heptahelical, G-protein-coupled receptors), and there are several isoforms of each class. HUVEC-CS express several P_2X receptors (P_2X_1, P_2X_2, P_2X_4 and P_2X_7) as well as a P_2Y receptor (P_2Y_2), which is typically associated with activation of phospholipase (PLC)-β (Murthy & Makhlouf 1998, Strassheim & Williams 2000, Tachibana *et al.* 2003).

A combination of ICC and Western blot analysis was used to demonstrate that Flt-1 and KDR (VEGF receptors, also known as VEGFR1 and VEGFR2/Flk-1 respectively) are present on the HUVEC-CS. This is significant because it implies that the HUVEC-CS should be able to respond to VEGF stimulation. In addition, HUVEC-CS express receptors for bFGF (flg-1), EGF (EGFR) and BK (B2R). They also express AT_1R and AT_2R, which are receptors for AII. Interestingly, AT_1R, B_2R, EGFR and KDR have all been localized to the caveolae along with eNOS, Gqα and Giα in various cell types (Smart *et al.* 1995, Mineo *et al.* 1996, DeWeerd & Leeb-Lundberg 1997, Kone 2000, Labrecque *et al.* 2003) Thus, HUVEC-CS probably can respond to ATP, VEGF, bFGF, EGF, BK and AII stimulation.

Ca^{2+} signaling is involved in a wide variety of endothelial cell processes, including vasodilator production (Tran *et al.* 2000) and mitogenesis (Schriffin 2001). In general, endothelial cells, such as rat brain capillary endothelial cells, primary human umbilical vein endothelial cells, bovine aortic endothelial cells and uterine artery endothelial cells derived from pregnant ewes, respond to Ca^{2+} mobilizing agonists such as ATP, BK and acetylcholine with a biphasic response (Bird *et al.* 2000, Tran *et al.* 2000, Di *et al.* 2001, Kimura *et al.* 2001, Gifford *et al.* 2003). HUVEC-CS are no exception to this trend. These cells respond to ATP in a time-dependent, dose-dependent and biphasic manner, which typically consists of an initial transient peak in the $[Ca^{2+}]_i$ followed by a prolonged sustained phase. Such an initial transient peak is most often caused by release of Ca^{2+} from intracellular stores. To determine whether this was occurring in the HUVEC-CS, the cells were treated with thapsigargin, an inhibitor of the Ca^{2+} ATPase on the ER. Treatment with this inhibitor causes the release of Ca^{2+} from the ER, triggering an influx of Ca^{2+} from the extracellular space through a process called capacitative Ca^{2+} entry (CCE). The ER cannot resequester the Ca^{2+} in the cytosol; consequently, the Ca^{2+} ATPase on the plasma membrane gradually pumps the Ca^{2+} out of the cell, and the intracellular Ca^{2+} pool is left depleted. This inhibitor is often used to determine whether an agonist causes Ca^{2+} mobilization from the ER because the agonist will be unable to initiate a

response if the store is already depleted. In the HUVEC-CS, ATP was unable to initiate a response after store depletion. This further implies that ATP induces Ca^{2+} mobilization through a P_2Y receptor. If ATP caused Ca^{2+} mobilization through a P_2X receptor, the influx would be from the extracellular space, and would therefore be unaffected by thapsigargin pretreatment. Rather, it seems likely that ATP is binding to a P_2Y receptor, leading to the activation of phospholipase C (PLC), which then hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 is released to the cytoplasm, binds to and activates IP_3 receptors (IP_3R) on the ER and releases Ca^{2+} from the ER, leading to a rapid transient rise in the $[Ca^{2+}]_i$ characteristic of the initial peak. However, further studies will be needed to verify that ATP does indeed work through a P_2Y receptor and that the inability of ATP to initiate a response after thapsigargin treatment was not simply due to desensitization.

The second phase of the biphasic Ca^{2+} response is generally produced by the influx of extracellular Ca^{2+}. In many cell types, the depletion of the intracellular stores triggers CCE through a putative store-operated channel (SOC). A second possibility is the opening of voltage-gated channels, particularly the L-type Ca^{2+} channels, through membrane depolarization. Historically, it was believed that voltage-gated channels were only in excitable cells. While this holds true for many endothelial cells, including primary HUVEC (Aono *et al.* 2000), some endothelial cells do contain functional L-type channels (Vinet & Vargas 1999, Yakubu & Leffler 2002). While the identity of SOC is still a matter of much debate and are very difficult to study, L-type channel function is easily identified. Two inhibitors of L-type channels and an L-type channel agonist were utilized in our studies. One inhibitor (verapamil) appeared to cause a slight but significant decrease in the ATP-induced response, which was not dose-dependent, and the agonist (BAYK8644) did not cause a Ca^{2+} response. Thus, it appears that L-type channels are absent or not functional on the HUVEC-CS.

Finally, the ability of AII, BK, EGF, bFGF and VEGF to mobilize Ca^{2+} was briefly evaluated. In single isolated cells, AII, BK and VEGF could induce Ca^{2+} mobilization in a subset of cells. When cell density was increased, more groups of cells responded to each of these agonists. In addition, at a higher cell density, EGF was able to induce a Ca^{2+} response. This implies that there may be communication between the cells at higher densities, or a contact-modulated change in cellular scaffold proteins and/or caveolae. Treatment with bFGF failed to initiate a response in either the single cells or groups of cells. While these same agonists were able to illicit other signaling responses at the same doses in HUVEC-CS as well as other endothelial cells (Bird *et al.* 2000, Di *et al.* 2001, Gifford *et al.* 2003), it may be that the dose of bFGF was not optimal to produce a Ca^{2+} response.

For many years, the dogma stated that eNOS could be activated only by an increase in $[Ca^{2+}]_i$. Over the past several years, a number of studies in a variety of endothelial cell models have shown that direct phosphorylation of eNOS by EKR 1/2 also affects NO production (Corson *et al.* 1996, Parenti *et al.* 1998, Fleming & Busse 2003). Furthermore, since vasodilation is an important endothelial cell function and because ERK 1/2 is involved in many other cell functions, the ability of agonists to stimulate ERK 1/2 activation was studied (Hood *et al.* 2003, Houle *et al.* 2003, Labrecque *et al.* 2003, Miura *et al.* 2003). ICC and Western blot analysis for the activated/phosphorylated form of ERK 1/2 were performed. ATP, bFGF, EGF and VEGF all cause a statistically significant rise in P-ERK 2, but AII and insulin do not. This rise in P-ERK 2 initiated by ATP, bFGF, EGF and VEGF is apparently via MEK, since it can be completely blocked by UO126.

Akt is another kinase that has been implicated in eNOS activation in several endothelial cell types (Dimmeler *et al.* 1999, Montagnani *et al.* 2001). However, Akt does not appear to be as important as ERK 1/2 in these acute experiments. Of the agonists tested, only EGF leads to increased Akt phosphorylation. Studies in primary HUVEC have demonstrated Akt phosphorylation after stimulation by insulin, bFGF or VEGF for 20 min or longer (Ido *et al.* 2002, Franklin *et al.* 2003, Wajih & Sane 2003). It is possible that the longer stimulation times used by these investigators could have resulted in a more robust and detectable Akt activation, so further studies may still be necessary to resolve this question.

Mitogenesis plays a fundamental role in the formation of new blood vessels as well as increasing the size of existing vessels. During pregnancy, this is particularly true of the uteroplacental vasculature and is in part due to an increase in a number of circulating growth factors. bFGF, EGF and VEGF, as well as ATP, caused an increase in cell proliferation in HUVEC-CS as measured by thymidine incorporation assays. The agonists tested which induced both a Ca^{2+} response and ERK 2 phosphorylation (ATP, EGF and VEGF) were able to promote mitogenesis. In addition, bFGF, which caused a rise in P-ERK 2 but could not cause any detectable rise in $[Ca^{2+}]_i$, did initiate thymidine incorporation. In contrast, AII, which mobilized Ca^{2+} but did not activate ERK 2, did not stimulate mitogenesis. This suggests that ERK 2 activation alone may be sufficient for an increase in cell proliferation. HUVEC-CS also appear to be among the cells in which Akt is not required for proliferation since agonists which did not activate Akt did cause thymidine incorporation. Nonetheless, the pattern of increased ERK 2 activation did not exactly parallel the trends of mitogenesis, so it is probable that either a threshold of activation must be reached or there is a complex interplay with several other signaling molecules involved in initiating mitogenesis in the HUVEC-CS.

NO, a key endothelium-derived relaxing factor, plays an important role in the maintenance of vascular tone and reactivity. The inability of endothelial cells to produce NO may underlie endothelial dysfunction. Indeed, endothelial dysfunction has been linked to virtually all cardiovascular diseases such as diabetes and atherosclerosis. Furthermore, eNOS knockout mice are incapable of normal vasodilation and have high blood pressure (Huang *et al.* 1995). In HUVEC-CS, ATP, EGF and VEGF all caused a significant increase in NO production, and this was paralleled by an ability to mobilize Ca^{2+}. Likewise, all of these agonists caused ERK 2 phosphorylation. Interestingly, AII, which can cause Ca^{2+} mobilization but not detectable ERK 2 phosphorylation, was unable to activate eNOS. In addition, bFGF, which caused ERK 2 activation but could not cause a detectable rise in the $[Ca^{2+}]_i$ even at an increased cell density, also failed to activate eNOS. Therefore, it is possible that both ERK 2 activation and Ca^{2+} mobilization are required for NO production in the HUVEC-CS, while neither alone is sufficient. The roles of Ca^{2+} and kinase may be unequal, however, given that the level of ERK 2 phosphorylation does not follow the same trends as eNOS activation, while the level of Ca^{2+} mobilization does. Thus, it may be that the rise in $[Ca^{2+}]_i$ determines the extent of eNOS activation as long as there is a minimal increase in ERK 2 phosphorylation. Furthermore, since EGF was the only agonist tested that caused Akt activation but without eNOS activity, it seems unlikely that Akt phosphorylation is required for acute eNOS activation in these cells. These data are generally consistent with the literature on primary HUVEC. One area of variability concerns the ability of AII, insulin and bFGF to cause eNOS activation; studies have shown AII, insulin and bFGF stimulation of NO production in primary HUVEC (Jozkowicz *et al.* 1999, Schena *et al.* 1999, Scharbrodt *et al.* 2004). However, other studies show that AII reduced NO bioavailability by promoting the generation of reactive oxygen species or by stimulating JNK and ERK 1/2, which in turn inhibit other pathways (in this case, IRS-1) (Desideri *et al.* 2003, Andreozzi *et al.* 2004). Thus, it appears that any change in experimental parameters that affects reactive oxygen species generation or kinase activation may alter eNOS activation, and it is quite possible that under different experimental conditions, all of the agonists tested may be capable of eNOS activation.

In summary, HUVEC-CS are a pure population of endothelial cells that actively proliferate and maintain established endothelial cell characteristics. Unlike some other endothelial cell lines, they do not require the addition of defined endothelial cell growth supplement to the media, nor do they have to be grown on Matrigel; furthermore, they propagate best on gelatin but can still be transferred to raw plastic for experimental use. Thus, they can be used to study growth factor-induced cellular functions such as mitogenesis, tube formation and migration. In addition, since these cells respond to VEGF and EGF, and particularly well to ATP to produce NO, they present a very useful model to study the regulation of NO production and its relationship to endothelial dysfunction. Furthermore, since these cells are of human origin, they can be used with human derived gene transformations, as well as human genomic and proteomic tools, a fact which may make this model system more attractive than commonly used endothelial cell models such as bovine aortic endothelial cells. Finally, because this subline is derived from human umbilical vein endothelial cells, further characterization of these cells may make them a consistent and useful tool of comparison with umbilical vein endothelial cells from diseased pregnancies.

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Note added in proof

Cells have been redeposited with ATCC.

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