Diabetes-induced activation of system y^+ and nitric oxide synthase in human endothelial cells: association with membrane hyperpolarization

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1. The activity of the human endothelial cell L-arginine transporter (system y^+) has been correlated with cGMP production (index of nitric oxide) and prostacyclin (PGI_2) release in umbilical vein endothelial cells cultured from normal or gestational diabetic pregnancies.

2. In non-diabetic and diabetic cells, transport of L-arginine was Na^+ and pH independent, inhibited by other cationic L-arginine analogues and unaffected by neutral amino acids.

3. Diabetes was associated with an increased V_{max} for saturable L-arginine transport ($4{\cdot}6 \pm 0{\cdot}13$ *vs.* $9{\cdot}9 \pm 0{\cdot}5$ pmol (μg protein)$^{-1}$ min^{-1}, $P < 0{\cdot}01$), but had no effect on initial rates of transport for L-serine, L-citrulline, L-leucine or 2-deoxyglucose.

4. In non-diabetic and diabetic cells, elevated K^+ resulted in a concentration-dependent inhibition in the initial rates of transport for L-arginine and the membrane potential-sensitive probe tetra[^{3}H]phenylphosphonium (TPP^+).

5. When resting membrane potential was measured using the whole-cell patch voltage clamp technique, diabetic cells were hyperpolarized ($-78 \pm 0{\cdot}3$ mV) compared with non-diabetic cells ($-70 \pm 0{\cdot}04$ mV, $P < 0{\cdot}04$). Accumulation of [^{3}H]TPP^+ was also increased in diabetic compared with non-diabetic cells.

6. Basal intracellular cGMP levels were elevated 2·5-fold in diabetic cells, and L-NAME (100 μM), an inhibitor of nitric oxide synthase, abolished basal cGMP accumulation in non-diabetic and diabetic cells.

7. Histamine (10 μM) had no effect on L-arginine transport but evoked significant increases in cGMP in non-diabetic and diabetic cells, which were completely inhibited by L-NAME but unaffected by superoxide dismutase.

8. Basal and histamine-stimulated PGI_2 release was decreased markedly in diabetic cells.

9. Our findings demonstrate that gestational diabetes is associated with phenotypic changes in fetal endothelial cells, which result in a membrane hyperpolarization, activation of the human endothelial cell L-arginine transporter (system y^+), elevation of basal nitric oxide synthesis and decreased PGI_2 production.

Abnormalities in vascular endothelial cell function occur early in the pathogenesis of insulin-dependent diabetes mellitus (reviewed by Bar, 1992; Cohen, 1993; Poston & Taylor, 1995). Nitric oxide (NO) has emerged as an important regulator of vascular tone and mediates vasodilatation by elevating cGMP levels in smooth muscle cells (reviewed by Knowles & Moncada, 1994). Under normal conditions, endothelial cells generate NO from L-arginine via a constitutive Ca^{2+}–calmodulin-dependent enzyme (cNOS) associated predominantly with the plasma membrane (Hecker, Mülsch, Bassenge, Föstermann & Busse, 1994), whereas a Ca^{2+}-insensitive isoform of NO synthase (iNOS) is induced in vascular endothelial and smooth muscle cells exposed to pro-inflammatory cytokines (Knowles & Moncada, 1994).

Although the response to vasodilators is generally normal in diabetic patients, the responses to vasoconstrictors are less well defined (reviewed by Poston & Taylor, 1995). The majority of animal studies, and some in man, suggest that basal NO release is abnormal in diabetes (Calver, Collier & Vallance, 1992; Langenstroer & Pieper, 1992; Elliott,

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Cockroft, Groop, Earle, Morocuti & Viberti, 1993; Tilton *et al.* 1993; Smulders *et al.* 1994). Of the few studies that have reported an enhanced production of NO in diabetes (Langenstroer & Pieper, 1992; Tilton *et al.* 1993; Smulders *et al.* 1994), some are based on the observation that aminoguanidine (an inhibitor of NO synthase and advanced glycosylation end products) reverses endothelial cell dysfunction in diabetes (Bucala, Tracey & Cerami, 1991; see review by Poston & Taylor, 1995). Most studies *in vitro* have not added L-arginine to the organ bath, which could lead to substrate depletion, if basal NO synthesis and L-arginine transport were enhanced. In addition to altered endothelial cell NO synthesis, the sensitivity of vascular smooth muscle cells to endothelium-derived NO may also be impaired in diabetic patients (Calver *et al.* 1992; McVeigh *et al.* 1992) and in animal models of diabetes (see Poston & Taylor, 1995).

To elucidate the cellular mechanisms mediating impaired endothelium-dependent relaxation in diabetes mellitus, we have investigated the L-arginine–NO signal transduction pathway and prostacyclin release in human cultured umbilical vein endothelial cells obtained from non-diabetic and gestational diabetic pregnancies.

Abstracts of part of this work have been published (Sobrevia, Jay, Morgan, Yudilevich & Mann, 1994*b*; Sobrevia, Yudilevich & Mann, 1994*c*).

METHODS

Patients and newborns

Paired experiments were conducted using umbilical cords from twenty-one full-term normal and twenty-one gestational diabetic pregnancies with normal spontaneous vaginal delivery (St Mary's Hospital, London, UK). All diabetic patients were normotensive, exhibited no albuminuria or glucosuria and had a mean glycosylated haemoglobin (HbA_1) plasma level of $7{\cdot}3 \pm 0{\cdot}4\%$. With the exception of two patients treated with insulin, gestational diabetic pregnancies were controlled by diet. Newborns did not present with symptoms of asphyxia and the mean newborn weight was 3975 g (range, 2810–4840 g) after 38 ± 2 weeks of gestation. The mean blood glucose concentration in the umbilical vein was $2{\cdot}1 \pm 0{\cdot}4$ mM.

Endothelial cell culture

Umbilical vein endothelial cells (HUVEC) were isolated by collagenase (0·5 mg ml^{-1}) digestion and cultured in medium 199 (M199) containing 5 mM D-glucose and supplemented with 10% fetal calf serum, 10% newborn calf serum, 5 mM glutamine, 100 i.u. ml^{-1} penicillin–streptomycin and 0·03 mg ml^{-1} gentamycin at 37 °C in a 5% CO_2 atmosphere (Mann, Pearson, Sheriff & Toothill, 1989). Confluent monolayers were trypsinized (trypsin–EDTA, 0·1–0·02%) and resuspended in serum-containing M199 supplemented with 20 μg ml^{-1} endothelial cell growth supplement (ECGS). For transport experiments and assays of cGMP and 6-keto-$PGF_{1\alpha}$, confluent second passage cells were resuspended in serum-containing M199 without ECGS and used 48–60 h later when cells had reached confluency. Endothelial cells were identified by their cobblestone morphology, ability to take up acetylated low-density lipoprotein and the absence of immunostaining for α-actin using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate (data not shown).

Measurements of cell protein, DNA and volume in confluent monolayers

Cell protein content was determined after addition of 100 μl Coomassie Blue protein reagent diluted 1:10, with bovine serum albumin (BSA) standards. Absorbances were measured at 620 nm in a Multiskan plate reader (Flow Laboratories, UK). Intracellular volume was determined from the distribution ratio of $[^3H_2O]$ at equilibrium using [^{14}C]mannitol as an extracellular marker. DNA content was assayed by adding sodium dodecylsulphate (10 μl, 10% in water) to each well, standards and blanks. After 5–10 min, 100 μl dye reagent (Hoechst 33258) was added and fluorescence (260 nm) read after 30 min in a Fluoroskan II microplate reader (Flow Laboratories)

Incorporation of L-[^{3}H]leucine and [^{3}H]thymidine

Actively replicating cells in the log phase of growth were pulsed with 5 μCi ml^{-1} L-[^{3}H]leucine or [^{3}H]thymidine. After 24 h, cells were rinsed twice with 500 μl warmed phosphate-buffered saline (PBS) and then exposed for 5 min to 200 μl of 5% trichloroacetic acid (TCA). TCA was removed and the monolayers were rinsed with 200 μl 90% methanol. Formic acid digests from each well were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting.

Endothelial cell transport of amino acids and 2-deoxyglucose

Confluent third-passage monolayers (2×10^4 cells per 96 well) were rinsed with warmed (37 °C) Krebs solution (mM: NaCl, 131; KCl, 5·6; $NaHCO_3$, 25; NaH_2PO_4, 1; D-glucose, 5; Hepes, 20; $CaCl_2$, 2·5; $MgCl_2$, 1; pH 7·4) and pre-incubated for 60 min at 37 °C in Krebs solution containing 100 μM L-arginine. The pre-incubation medium was removed and cells incubated for specified times (0–60 min) with Krebs solution (+ 100 μM L-arginine) containing L-[^{3}H]arginine and D-[^{14}C]mannitol (extracellular reference). In some experiments initial rates of L-arginine transport (1 min) were measured in cells incubated with increasing concentrations of KCl (5·5–131 mM). In experiments where extracellular KCl was increased, NaCl was decreased isosmotically. In other experiments Krebs solution was depleted of Na^+ by isosmotic replacement with choline chloride, choline bicarbonate and KH_2PO_4, or adjusted to pH 6·0, 7·0, 7·4, 8·0 by addition of HCl (0·5 N) or NaOH (0·5 N) to the Krebs medium. As in the case of L-arginine, transport (100 μM) of radiolabelled L-serine, L-citrulline, L-leucine and 2-deoxy-D-glucose was compared in non-diabetic and diabetic cells. Tracer uptake was terminated by removal of the uptake medium 1 s before rinsing the monolayer three times with 200 μl ice-cold stop solution (Krebs buffer containing 10 mM unlabelled substrate). Radioactivity in formic acid cell digests was determined by liquid scintillation counting, and uptake corrected for [^{14}C or ^{3}H]mannitol d.p.m. in the extracellular space.

Selectivity and kinetics of L-arginine transport

Inhibition of L-arginine uptake by putative competitor amino acids and L-arginine analogues was tested by incubating endothelial cell monolayers with Krebs solution containing 100 μM L-[^{3}H]arginine in the absence or presence of a given inhibitor (0·03–5 mM). Initially, cross-inhibition experiments were performed at a fixed inhibitor concentration of 1 mM. We then examined further the concentration-dependent (0·03–5 mM) inhibition of L-arginine transport by L-homoarginine, 2-methylaminoisobutyric acid (MeAIB) and the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA).

Kinetic experiments were performed using cells incubated for 1 min with Krebs solution containing Na^+ and increasing concentrations of L-arginine (0·015–1 mM). Data were analysed using the computer programmes Enzfitter and Ultra Fit (Elsevier, Biosoft).

Measurement of tetra[^{3}H]phenylphosphonium (TPP^+) uptake

Endothelial cells were grown to confluency in 96-well microtitre plates. The culture medium was aspirated, and the monolayers immediately washed and incubated with Krebs medium containing 100 μM L-arginine for 60 min (see above). After a final wash, 50 μl of Krebs solution containing 11 nM [^{3}H]TPP^+ (0·12 μCi ml^{-1}) and increasing concentrations of KCl (5·5–131 mM) were added to the wells and, as previously described (Schilling, 1989), uptake of [^{3}H]TPP^+ was measured over various time intervals (0–120 s). Tracer uptake was terminated by rinsing the monolayer three times with 200 μl ice-cold stop solution and radioactivity associated with the cells was determined by liquid scintillation counting (see above).

Resting membrane potential measured by whole-cell patch voltage clamp

Resting membrane potential was measured by the patch clamp technique in the whole-cell configuration. Subconfluent endothelial cells grown on 35 mm plastic dishes were incubated in Krebs solution (see above) and transferred to the stage of an inverted microscope (Zeiss IM 35). Recordings were performed using an EPC-7 amplifier (List Medical, Darmstadt, Germany). Patch pipettes were filled with the following solution (mM): KCl, 135; $CaCl_2$, 0·2; $MgCl_2$, 1·6; Hepes acid, 10; EGTA, 2; K-ATP, 2·5; Li-GTP, 0·2 (pH adjusted to 7·3 with KOH); and had a resistance of 4–6 MΩ. On obtaining the whole-cell configuration, the amplifier was switched from voltage clamp to current clamp mode. The membrane potential was measured for at least 1 min, and only stable recordings were included in the analysis.

Basal and histamine-stimulated cGMP levels and prostacyclin release

Confluent monolayers in 24-well plates were pre-incubated for 15 min with Krebs solution (37 °C) containing 100 μM L-arginine and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0·5 mM). The pre-incubation medium was removed and 500 μl Krebs solution containing IBMX (with or without 10 μM histamine) was added to the wells for a further 5 min (37 °C). The supernatant was removed for analysis of PGI_2 release by radioimmunoassay of its stable metabolite 6-keto-$PGF_{1\alpha}$ (Toothill, Needham, Gordon & Pearson, 1989). Cells were then placed on ice and incubated with 0·1 N HCl (1 ml per well, 60 min) and 800 μl HCl cell extract was stored at −20 °C for radioimmunoassay of cGMP following acetylation (Bogle, Coade, Moncada, Pearson & Mann, 1991). The effects of N^G-nitro-L-arginine methyl ester (L-NAME) or superoxide dismutase (SOD) on cGMP levels were assessed by pre-incubating cells for 15 min with Krebs solution containing 100 μM L-NAME or 50 i.u. ml^{-1} SOD, and then for a further 5 min with these agents in the absence or presence of 10 μM histamine.

Materials

Newborn and fetal calf serum and all other reagents were purchased from Sigma. Collagenase Type II from *Clostridium histolyticum* was from Boehringer Mannheim (Germany) and Bradford protein reagent from BioRad Laboratories (Herts, UK). L-[2,3-^{3}H]-Arginine (36·1 Ci mmol^{-1}), L-[4,5-^{3}H(*N*)]-leucine (70 Ci mmol^{-1}), [methyl-^{3}H]thymidine (5 Ci mmol^{-1}), L-[carbamoyl-^{14}C]citrulline (54·3 mCi mmol^{-1}), D-[1-^{14}C or ^{3}H]-mannitol (49·3 mCi mmol^{-1} or 56 Ci mmol^{-1}) and tetraphenylphosphonium bromide[phenyl-^{3}H] (37 Ci mmol^{-1}) were obtained from New England Nuclear (Dreieich, Germany) and 2-deoxy-D-[2,6-^{3}H]-glucose (42 Ci mmol^{-1}) and L-[3-^{3}H]-serine (32 Ci mmol^{-1}) from Amersham International (UK). 3′,5′-cyclic GMP-TME, [tyrosine-^{125}I] from ICN (UK) and [^{125}I]6-keto-$PGF_{1\alpha}$ from Advanced Magnetics Inc. (UK).

Statistics

Values are expressed as means ± S.E.M., where n indicates the number of different endothelial cell cultures with four to six replicate measurements. Statistical analyses were performed using Student's unpaired t test and $P < 0·05$ was considered significant.

RESULTS

At confluence, total protein, DNA, cell number and cell volume were similar in normal and diabetic cells (Table 1). Incorporation of L-[^{3}H]leucine and [^{3}H]thymidine in actively replicating cells was reduced in diabetic endothelium (Table 1), confirming previous findings that endothelial cells from diabetic pregnancies reach confluency more slowly than normal cells (Karbowsky, Bauch & Schneider, 1989; Sobrevia, Jarvis & Yudilevich, 1994*a*).

Effects of diabetes on L-arginine transport

In normal and diabetic cells L-arginine transport was linear for up to 1 min, and removal of Na^+, or changes in extracellular pH, had no significant effect on L-arginine transport in either cell type (data not shown). Overall transport rates for L-arginine (0·015–1 mM) in both cell types were fitted best by a Michaelis–Menten equation plus a linear non-saturable component. The saturable components for L-arginine transport are shown in Fig. 1*A*. Diabetes

Table 1. Effect of gestational diabetes on human umbilical vein endothelial cell properties

	Protein content (μg (10^4 cells)$^{-1}$)	DNA content (μg (10^4 cells)$^{-1}$)	Cell number (cells (cm^2)$^{-1}$)	Cell volume (pl (cell)$^{-1}$)	L-[^{3}H]Leucine incorporation (d.p.m. (10^4 cells)$^{-1}$)	[^{3}H]Thymidine incorporation (d.p.m. (10^4 cells)$^{-1}$)
Non-diabetic	2·8 ± 0·2	1·2 ± 0·03	42 456 ± 1365	1·7 ± 0·3	29 614 ± 3344	5759 ± 915
Diabetic	2·6 ± 0·3	1·2 ± 0·04	44 869 ± 1378	1·9 ± 0·5	4427 ± 862*	1104 ± 302*

Protein, DNA content and cell number were determined in confluent cell monolayers. Incorporation of L-[^{3}H]leucine and [^{3}H]thymidine was measured as described in Methods. Values are the means ± S.E.M. of 12 experiments. The mean cell volume measurements were obtained from 6 experiments; * $P < 0·01$ relative to values in normal cells.

Table 2. Effects of gestational diabetes on the kinetics of L-arginine transport

	K_m (μM)	V_{max} (pmol (μg protein)$^{-1}$ min^{-1})	K_D (pmol (μg protein)$^{-1}$ min^{-1} μM^{-1})	V_{max}/K_m
Non-diabetic	101 ± 10	4·6 ± 0·13	0·0072 ± 0·0005	0·046 ± 0·01
Diabetic	129 ± 18	9·9 ± 0·5*	0·0157 ± 0·0017*	0·077 ± 0·03

Saturable L-arginine transport kinetics were measured as described in Methods. Values are the means ± S.E.M. of 4–8 experiments; * $P < 0·01$ relative to normal cells.

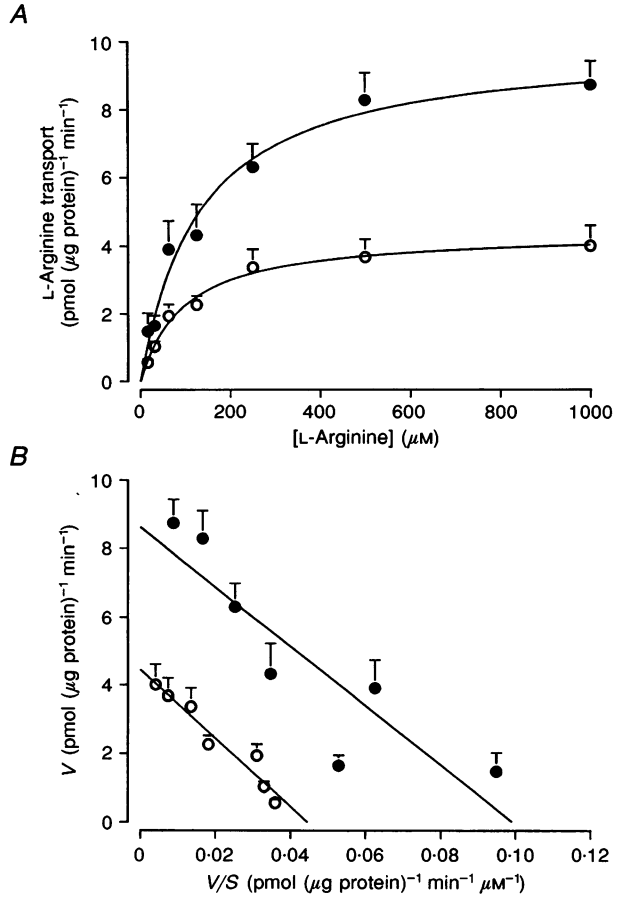

Figure 1. L-Arginine transport in human umbilical vein endothelial cell monolayers isolated from non-diabetic (○) and gestational diabetic (●) pregnancies
Monolayers were incubated with L-[^{3}H]arginine at 37 °C and initial rates of total L-arginine influx (0·015–1 mM) were determined over 1 min. Michaelis–Menten hyperbolae plus a linear non-saturable component were fitted (see Methods) to each set of mean influx values weighted for the reciprocal of the respective standard error.
A, initial rates of saturable L-arginine influx.
B, Eadie–Hofstee plots of initial rates of saturable L-arginine transport against influx/[L-arginine]. Weighted regression analyses revealed the presence of a single high-affinity transport system in non-diabetic ($r = 0·98$) and diabetic ($r = 0·93$) cells. K_m values were 101 ± 10 and 87 ± 12 μM in normal and diabetic cells, respectively. Values are the means ± S.E.M. of experiments in 8 different cell cultures.

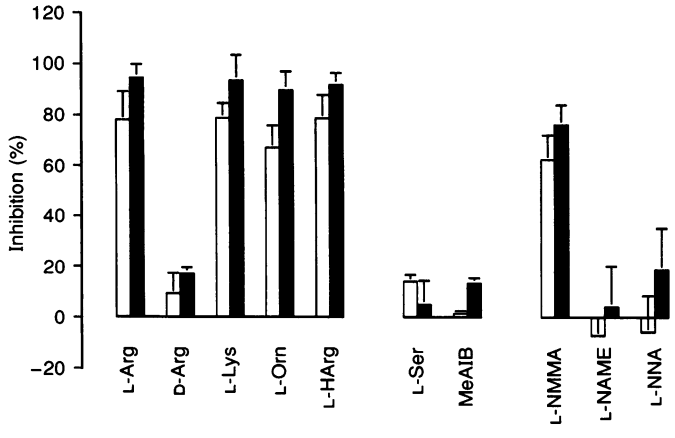

Figure 2. Specificity of L-arginine transport in non-diabetic and diabetic endothelial cells
Saturable transport of L-[^{3}H]arginine (100 μM, 1 min) was measured in the presence of putative competitor amino acids (1 mM) and NO synthase inhibitors (100 μM). Cross-inhibition studies were performed in non-diabetic (□) and diabetic (■) cells in the presence of Na^+. Data are expressed as the percentage inhibition of saturable L-arginine influx measured in the absence of an inhibitor. Abbreviations denote standard nomenclature: L- and D-arginine (L-Arg, D-Arg), L-lysine (L-Lys), L-ornithine (L-Orn), L-homoarginine (L-HArg), L-serine (L-Ser), 2-methylaminoisobutyric acid (MeAIB), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NNA). Values are the means ± S.E.M. of experiments in 5 different cell cultures.

Table 3. Effect of gestational diabetes on amino acid and 2-deoxyglucose transport

		Transport (pmol (μg protein)$^{-1}$ min^{-1})	
	Time of uptake (s)	Non-diabetic	Diabetic
L-Citrulline	30	2·1 ± 0·2	3·1 ± 0·9
L-Leucine	60	5·6 ± 0·8	6·4 ± 0·9
L-Serine	30	1·9 ± 0·6	2·8 ± 0·7
2-Deoxy-D-glucose	30	3·5 ± 0·2	4·1 ± 0·7
Adenosine †	5	45·5 ± 5·3	18·5 ± 2·9*

Overall rates of transport for different radiolabelled substrates (100 μM) were determined in non-diabetic and diabetic endothelial cells incubated with Krebs solution (37 °C) for the specified periods. Values in the present experiments are the means ± S.E.M. of 4–11 experiments; * $P < 0·002$ relative to normal cells. † For comparison, data for adenosine (10 μM) have been recalculated from Fig. 2 in Sobrevia *et al.* 1994*a*.

increased the V_{max} for L-arginine transport 2·2-fold, without altering the apparent K_m (Table 2). Although the value of K_D (non-saturable component) was elevated in diabetic compared with non-diabetic cells, we could not fit the overall transport data with a transport rate equation for two saturable systems acting in parallel. Eadie–Hofstee analyses of transport were linear (Fig. 1*B*), suggesting the presence of a single high-affinity transport site for L-arginine in both cell types. Stimulation of L-arginine transport by diabetes appeared to be selective as transport of L-serine, L-leucine, L-citrulline and 2-deoxyglucose were not altered significantly (Table 3).

To further exclude the possibility that diabetes altered the specifity of L-arginine transport in human umbilical vein endothelial cells, we screened the inhibitory effects of putative competitor amino acids (1 mM), and the data for non-diabetic and diabetic cells are summarized in Fig. 2. L-Arginine transport was stereospecific and inhibited markedly by other cationic amino acids (L-lysine, L-ornithine and L-homoarginine), and the potent inhibitor of NO synthase, L-NMMA. In contrast, the neutral amino acids, L-serine and MeAIB, and the neutral NO synthase inhibitors, N^G-nitro-L-arginine (L-NNA) and its methyl ester, L-NAME, were poor inhibitors. In subsequent

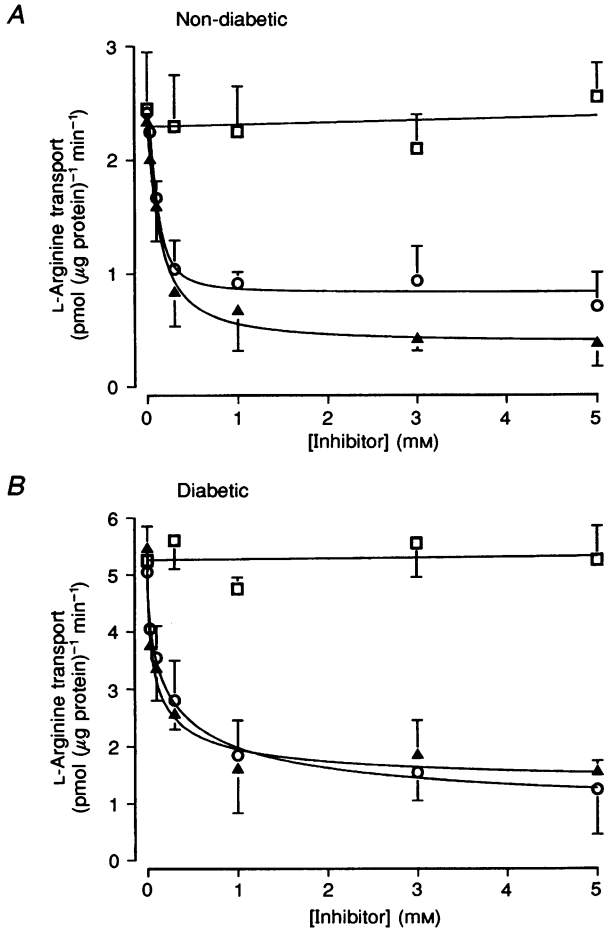

Figure 3. Inhibition of L-arginine transport by L-NMMA, L-homoarginine and MeAIB

Transport of L-[^{3}H]arginine (100 μM, 1 min) was measured in the absence or presence of increasing concentrations (0·03–5 mM) of L-NMMA (○), L-homoarginine (▲), or 2-methylaminoisobutyric acid (□) in non-diabetic (*A*) or in diabetic (*B*) cells. Note that the basal rates of L-arginine transport were elevated in diabetic cells. Values are the means ± S.E.M. of experiments in 5 different cell cultures.

Table 4. Effect of gestational diabetes on L-arginine transport, TPP^+ uptake and resting membrane potential in human umbilical vein endothelial cells in culture

	L-Arginine transport (pmol (μg protein)$^{-1}$ min^{-1})	TPP^+ uptake (pmol (10^6 cells)$^{-1}$ min^{-1})	Membrane potential (mV)
Non-diabetic	4·1 ± 0·4	0·52 ± 0·2	−70·0 ± 0·4
Diabetic	6·8 ± 0·8	0·75 ± 0·03	−78·2 ± 0·3
P	0·018	0·029	0·035

Initial rates for saturable L-arginine transport and TPP^+ uptake were measured in confluent monolayers, and the resting membrane potential was measured using the whole-cell patch clamp technique as described in Methods. Values are the means ± S.E.M. of 3–6 experiments; *P* values are relative to non-diabetic cells.

experiments we examined further the concentration-dependent inhibition of saturable L-arginine transport by L-homoarginine, L-NMMA and MeAIB (0·03–5 mM). As shown in Fig. 3, MeAIB was an ineffective inhibitor in both non-diabetic and diabetic endothelial cells. In contrast, the cationic L-arginine analogues, L-homoarginine and L-NMMA, were effective inhibitors in both cells types (Fig. 3). The calculated apparent K_i values (concentrations giving half-maximal inhibition) for L-homoarginine (non-diabetic, 87 ± 16 μM; diabetic, 148 ± 44 μM) and L-NMMA (non-diabetic, 93 ± 30 μM; diabetic, 89 ± 10 μM) were in the same range as the corresponding K_m for L-arginine (Table 2). L-Arginine transport was not affected by equimolar concentrations of D-mannitol (data not shown), confirming that the observed inhibitions were not due to osmotic effects.

Effects of diabetes on the membrane potential and TPP^+ influx

In patch clamp experiments, the resting membrane potential in diabetic cells was hyperpolarized compared with non-diabetic cells (Table 4). In parallel experiments, initial rates of TPP^+ influx were also increased significantly in diabetic compared with non-diabetic cells (see Fig. 4).

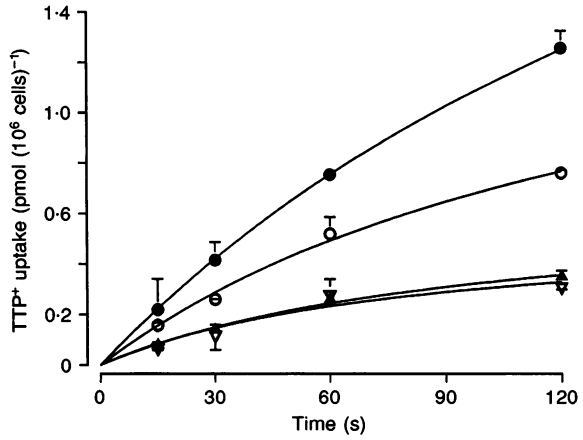

Figure 4. Effects of diabetes and elevated K^+ on uptake of the membrane potential-sensitive probe TPP^+

The time course of [^{3}H]TPP^+ uptake was determined in non-diabetic (open symbols) and diabetic (filled symbols) endothelial cell monolayers cultured in normal (5·5 mM, ○, ●) or elevated (131 mM, ▽, ▲) K^+. Values are the means ± S.E.M. of experiments in 4 different cell cultures.

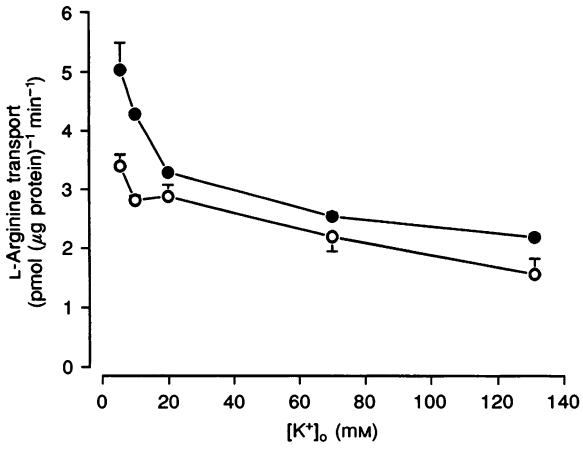

Figure 5. Effects of elevated K^+ on L-arginine transport in non-diabetic and diabetic endothelial cells

Initial rates of L-arginine transport (100 μM, 1 min) were determined in non-diabetic (○) and diabetic (●) cells exposed to increasing concentrations of extracellular K^+ (5·5–131 mM). Values are the means ± S.E.M. of experiments in 4 different cell cultures.

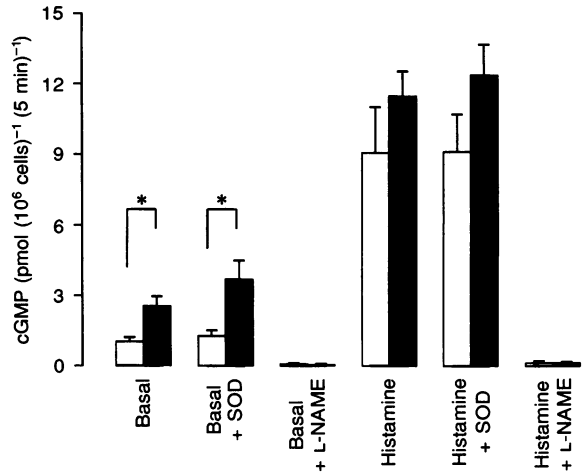

Figure 6. Basal and histamine-stimulated cGMP levels in non-diabetic and diabetic endothelial cells

Non-diabetic (□) and diabetic (■) cells were initially pre-incubated with 0·5 mM IBMX (15 min, 37 °C), and HCl cell extracts were then stored at −20 °C for radioimmunoassay of cGMP levels. cGMP accumulation was measured in the absence or presence of histamine (10 μM, 5 min, plus 0·5 mM IBMX) and/or superoxide dismutase (SOD, 50 i.u. ml^{-1}). Basal and histamine-stimulated cGMP levels were also measured in the presence of 100 μM L-NAME. * $P < 0·03$ relative to corresponding control values in non-diabetic cells. Values are the means ± S.E.M. of experiments in 7 different cell cultures.

These two independent experimental measurements suggest that the membrane potential in diabetic endothelial cells is hyperpolarized, a finding consistent with the elevated transport rates measured for the cationic substrate L-arginine in diabetic cells (Table 4).

Effects of elevated extracellular K^+ on L-arginine and TPP^+ influx

To determine whether diabetes *per se* alters the sensitivity of TPP^+ and L-arginine influx to changes in extracellular K^+, we compared initial rates of transport in non-diabetic and diabetic cells exposed to increasing concentrations of extracellular K^+. TPP^+ influx was inhibited by elevated K^+ (Fig. 4), and in paired experiments, initial rates of L-arginine transport were also decreased by increasing extracellular concentrations of K^+ (Fig. 5).

Effects of diabetes on basal and histamine-stimulated NO and PGI_2 release

Stimulation of normal or diabetic endothelial cells with 10 μM histamine had no effect on L-arginine transport, although L-arginine influx remained significantly elevated in diabetic endothelial cells (data not shown). As shown in Fig. 6, basal cGMP levels were 2·5-fold higher ($P < 0·01$) in diabetic cells (2·56 ± 0·40 pmol $(10^6 \text{ cells})^{-1}$ $(5 \text{ min})^{-1}$) than in non-diabetic cells (1·02 ± 0·18 pmol $(10^6 \text{ cells})^{-1}$ $(5 \text{ min})^{-1}$), and were not altered significantly following treatment of cells with superoxide dismutase (50 i.u. ml^{-1} for 5 min). Pretreatment of cells with the NO synthase inhibitor L-NAME (100 μM), which does not inhibit L-arginine transport in porcine aortic endothelial cells (Bogle, Moncada, Pearson & Mann, 1992) or human umbilical vein endothelial cells (Fig. 2), resulted in a complete inhibition of basal cGMP accumulation in both cell types (Fig. 6). Histamine (10 μM, 5 min) elevated cGMP levels in non-diabetic cells (9·08 ± 1·94 pmol $(10^6 \text{ cells})^{-1}$ $(5 \text{ min})^{-1}$) and diabetic cells (11·5 ± 1·05 pmol $(10^6 \text{ cells})^{-1}$ $(5 \text{ min})^{-1}$). Treatment with superoxide dismutase had no effect on histamine-stimulated cGMP levels, whereas L-NAME abolished agonist-induced increases in cGMP (Fig. 6).

Basal production of prostacyclin (PGI_2) was reduced in diabetic endothelial cells (Fig. 7). Although both non-diabetic and diabetic cells responded to histamine stimulation (10 μM), maximal rates of agonist-induced PGI_2 release were significantly lower in diabetic cells. Basal and histamine-stimulated PGI_2 release was unaffected by L-NAME (100 μM).

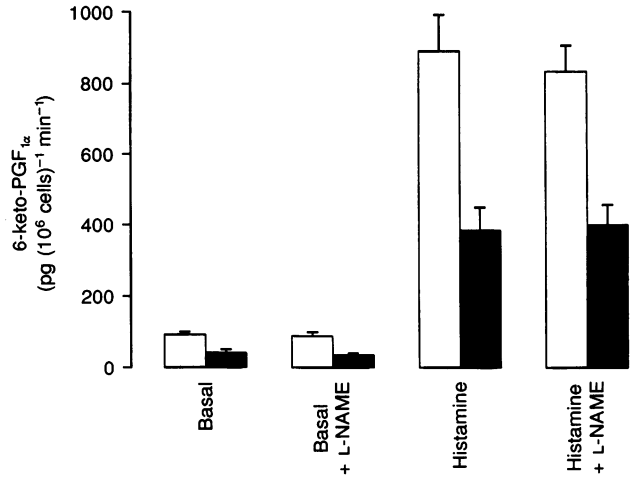

Figure 7. Basal and histamine-stimulated rates of PGI_2 release in non-diabetic and diabetic endothelial cells

As release of PGI_2 and cGMP accumulation (Fig. 6) were measured in the same experiments, cells were incubated with Krebs solution containing 0·5 mM IBMX in the absence or presence of 100 μM L-NAME. The supernatants from the different wells were stored at 4 °C for radioimmunoassay of 6-keto-$PGF_{1\alpha}$. Values are the means ± S.E.M. of experiments in 7 different cell cultures; $P < 0·05$ relative to values in non-diabetic cells.

DISCUSSION

The present study demonstrates that activity of the L-arginine transporter (system y^+) is upregulated selectively in human endothelial cells isolated from gestational diabetic pregnancies. The enhanced rate of L-arginine transport in cultured diabetic endothelial cells was accompanied by an increased basal release of NO and a reduced basal production of PGI_2. Although histamine-stimulated NO release was similar in both cell types, PGI_2 release was decreased by 50% in diabetic cells. In addition, diabetic endothelial cells were characterized by other phenotypic changes, in particular, reduced rates of L-leucine and thymidine incorporation.

System y^+ has previously been shown to mediate L-arginine transport in endothelial cells (Bogle *et al.* 1992; Greene, Pacitti & Souba, 1993; Bussolati, Sala, Astorri, Rotoli, Dall'Asta & Gazzola, 1993). Our present findings confirm this and demonstrate that the same system exists, with a higher maximal activity, in diabetic endothelial cells. The K_m value for L-arginine in umbilical vein endothelial cells (0·1 mM) is lower than values (0·3 and 3·9 mM) estimated in cultured bovine pulmonary artery endothelial cells, in which three saturable transport systems were described for L-arginine (Greene *et al.* 1993). These discrepancies may well reflect inherent characteristics of venous and pulmonary artery endothelial cells or species differences. The kinetic properties of L-arginine transport in umbilical vein endothelial cells resemble those of system y^+ (MCAT-1, murine cationic amino acid transporter), recently cloned and expressed in *Xenopus* oocytes (Kim, Closs, Albritton & Cunningham, 1991; Wang, Kavanaugh, North & Kabat, 1991). Our kinetic experiments were performed over a wide range of substrate concentrations, but we did not detect the activity of any other higher affinity system, such as y^+L ($K_m = 10$–20 μM), known to mediate entry of L-lysine and neutral amino acids in human erythrocytes (Devés, Chavez & Boyd, 1992). Although it would be of interest to examine L-arginine transport at low substrate concentrations (~1 μM) in the presence of *N*-ethylmaleimide (an inhibitor of system y^+ activity in erythrocytes; Devés, Angelo & Chavez, 1993), it seems likely that system y^+L would be saturated at plasma L-arginine concentrations.

Inhibition studies of L-arginine transport in non-diabetic and diabetic umbilical vein endothelial cells (Fig. 2) revealed that D-arginine was a poor inhibitor, whereas L-lysine, L-ornithine or L-homoarginine reduced transport significantly. The apparent K_i values determined for L-homoarginine and the cationic NO synthase inhibitor L-NMMA were similar to the K_m value for L-arginine transport (Table 2). The insensitivity of L-arginine transport to 10-fold excess concentrations of MeAIB, L-serine and the neutral NO synthase inhibitors L-NNA and L-NAME, indicates that these amino acids are not transported effectively by system y^+. L-NMMA has positively-charged guanido and amidino groups ($pK_a \approx 13$) which render this L-arginine analogue strongly basic, whilst L-NNA and L-NAME have pK_a values close to zero and would be neutral at physiological pH (see Knowles & Moncada, 1994; Baydoun & Mann, 1994). We conclude, therefore, that transport of L-arginine and other cationic amino acids, including L-NMMA, in both non-diabetic and diabetic endothelial cells is mediated by the relatively high-affinity, Na^+-independent and pH-insensitive system y^+ (MCAT-1).

In endothelial cells system y^+ transport activity is sensitive to changes in extracellular K^+, suggesting that L-arginine influx is influenced by alterations in membrane potential (Bussolati *et al.* 1993; Kavanaugh, 1993). We have confirmed and extended these findings by determining the resting membrane potential in non-diabetic and diabetic endothelial cells using the whole-cell patch clamp technique. The resting membrane potential was hyperpolarized in diabetic compared with non-diabetic cells. In parallel experiments we also demonstrated that initial rates of uptake of the membrane potential-sensitive probe TPP^+ were increased significantly in diabetic cells, a finding consistent with our direct measurements of membrane potential. Depolarization of the endothelial cell membrane with elevated K^+ significantly reduced TPP^+ uptake and L-arginine transport in both cell types. To our knowledge, these findings provide the first direct evidence that human endothelial cells obtained from diabetic pregnancies can sustain a small membrane hyperpolarization during prolonged cell culture, but it leaves open the question of whether or not the membrane potential in the endothelium of the diabetic umbilical vein is altered *in situ*. Although the membrane hyperpolarization may, in part, account for the elevated transport rates of L-arginine in diabetic endothelial cells, we cannot exclude the additional influence of changes in the number of functional transporters, decreased degradation of membrane carriers and/or increased *trans*-stimulation by intracellular substrates.

Unlike our findings with L-arginine, endothelial cell transport of neutral amino acids was unaffected by diabetes. In this context it is interesting that transport of neutral amino acids via the Na^+-dependent system A is reduced in microvillous membrane vesicles isolated from placentae of diabetic women (Kuruvilla, D'Souza, Glazier, Mahendran, Maresh & Sibley, 1994).

There are divergent reports that basal synthesis of endothelium-derived NO may be either increased or decreased in diabetic patients (see Poston & Taylor, 1995). Our results demonstrate a significantly elevated basal accumulation of cGMP (index of NO release) by fetal endothelial cells isolated from diabetic mothers (Fig. 6). Although Hattori, Kawasaki, Abe & Kanno (1991) have reported that superoxide dismutase (SOD) restores impaired endothelium-dependent relaxation, we found that SOD had no significant effect on basal or histamine-stimulated cGMP

accumulation. It is plausible that the increased basal synthesis of NO is a consequence of the elevated rate of L-arginine transport, though we have no direct evidence. Furthermore, inhibition of cGMP accumulation by L-NAME, under both basal and histamine-stimulated conditions, did not reduce the increased rate of L-arginine transport in diabetic cells. Under the conditions of the present experiments, histamine had no effect on L-arginine transport in either cell type, suggesting that the enhanced accumulation of cGMP in response to histamine may not necessarily be coupled directly to L-arginine transport activity. It is also worth noting that inhibition of cGMP accumulation by L-NAME, under both basal and histamine-stimulated conditions, did not reduce the increased rates of L-arginine transport in diabetic cells. However, rates of histamine-stimulated NO production were similar in non-diabetic and diabetic cells, suggesting that levels of NO synthase were similar in these two cell types. These findings would argue against the proposal that endothelial cell dysfunction *in vitro* is due to the depletion of L-arginine from arterial rings as a result of its omission from an organ bath. Under the same cell culture conditions, we observed that basal and histamine-stimulated PGI_2 release were inhibited in diabetic cells, confirming an earlier study in umbilical vein endothelial cells obtained from non-diabetic pregnancies (Karbowski *et al.* 1989).

The present findings, together with our previous report that transport and metabolism of adenosine are significantly impaired in diabetic endothelial cells (Sobrevia *et al.* 1994*a*), provide unique markers for the altered metabolic status of fetal endothelial cells in diabetes. Maternal hyperglycaemia usually leads to fetal hyperglycaemia and hyperinsulinaemia, which are associated with an increased intrauterine fetal growth (Pedersen, 1954; see review by Dornhorst & Beard, 1993). In our study umbilical vein blood glucose levels at delivery were only 2·1 mM, yet diabetic endothelial cells in culture expressed phenotypic changes compared with non-diabetic cells. Nevertheless, extrapolation of our findings to adult non-pregnant diabetic patients should be treated with caution.

Although the rates of L-arginine transport and basal NO synthesis, based on accumulation of cGMP, were paradoxically increased in human endothelial cells isolated from gestational diabetic pregnancies, it is likely that dysfunction in diabetic blood vessels may occur due to enhanced quenching of NO by oxygen-derived free radicals, advanced glycosylation end products and/or a decreased sensitivity of vascular smooth muscle to endothelium-derived NO. Our findings establish that gestational diabetes induces phenotypic changes in human fetal endothelium which are associated with a membrane hyperpolarization, activation of the L-arginine transporter (system y^+–MCAT-1), elevation of basal NO synthesis and decreased basal and histamine-stimulated PGI_2 release.

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