Intrauterine Growth Retardation Is Associated With Reduced Activity and Expression of the Cationic Amino Acid Transport Systems \( y^+/h\text{CAT}-1 \) and \( y^+/h\text{CAT}-2B \) and Lower Activity of Nitric Oxide Synthase in Human Umbilical Vein Endothelial Cells

Paola Casanello, Luis Sobrevia

Abstract—Intrauterine growth retardation (IUGR) is associated with vascular complications leading to hypoxia and abnormal fetal development. The effect of IUGR on \( l\)-arginine transport and nitric oxide (NO) synthesis was investigated in cultures of human umbilical vein endothelial cells (HUVECs). IUGR was associated with membrane depolarization and reduced \( l\)-arginine transport (\( V_{\text{max}} = 5.8 \pm 0.2 \) versus 3.3 \( \pm \) 0.1 pmol/\( \mu \)g protein per minute), with no significant changes in transport affinity (\( K_m = 159 \pm 15 \) versus 137 \( \pm \) 14 \( \mu \)mol/L). \( l\)-Arginine transport was trans-stimulated (8- to 9-fold) in cells from normal and IUGR pregnancies. IUGR was associated with reduced production of \( l\)-\[^{3}H\]citrulline from \( l\)-\[^{3}H\]arginine, lower nitrite and intracellular \( l\)-arginine, \( l\)-citrulline, and cGMP. IUGR decreased hCAT-1 and hCAT-2B mRNA, and increased eNOS mRNA and protein levels. IUGR-associated inhibition of \( l\)-arginine transport and NO synthesis, and membrane depolarization were reversed by the NO donor \( S\)-nitroso-\( N\)-acetyl-\( L\),\( D\)-penicillamine. In summary, endothelium from fetuses with IUGR exhibit altered \( l\)-arginine transport and NO synthesis (\( l\)-arginine/NO pathway), reduced expression and activity of hCAT-1 and hCAT-2B and reduced eNOS activity. Alterations in \( l\)-arginine/NO pathway could be critical for the physiological processes involved in the etiology of IUGR in human pregnancies. (Circ Res. 2002;91:127-134.)

Key Words: \( l\)-arginine • intrauterine growth retardation • nitric oxide • human • endothelium

Intrauterine growth retardation (IUGR) is associated with prenatal disturbances, including prematurity and fetal asphyxia. Fetal growth and development depend on fetal tissue oxygenation and substrate delivery. The human fetoplacental circulation exhibits a low vascular resistance and lacks autonomic innervation. Thus, circulating and locally released vasoactive molecules are therefore likely to be involved in the control of fetoplacental hemodynamics. The release of local vasoactive molecules, such as nitric oxide (NO), from endothelium maintains appropriate placental blood flow, fetal nutrition, and oxygenation leading to normal fetal development and growth.

Endothelial NO synthesis results from conversion of \( l\)-arginine into \( l\)-citrulline by the \( Ca^{2+}/\text{calmodulin}-dependent \) NO synthase (eNOS), a process associated with \( l\)-arginine transport via system \( y^+/\text{CATs} \) (Cationic Amino Acid Transporters) in human umbilical vein endothelial cells (HUVECs). \( l\)-Arginine transport is preferentially mediated by system \( y^+/\text{CAT}-1 \), a \( Na^+ \)-independent, high-affinity transport system (\( K_m \approx 100 \) \( \mu \)mol/L), sensitive to changes in membrane potential in HUVECs.

Long-term inhibition of NO synthase mimics IUGR in gravid rats, and eNOS-targeted mutagenesis is associated with fetal growth retardation in mouse. Other studies show that eNOS protein levels are reduced in rat placental microvasculature of IUGR pregnancies. Furthermore, \( l\)-arginine supplementation prevents fetal growth retardation in animal models of IUGR, and \( l\)-arginine infusion improved vasorelaxation in IUGR pregnant women with increased uterine resistance. Therefore, \( l\)-arginine/NO signaling pathway could play a crucial role in IUGR pregnancies.

We characterized \( l\)-arginine transport and NO synthesis in HUVECs from normal pregnancies or pregnancies complicated with IUGR. IUGR is associated with reduced activity of \( l\)-arginine/NO pathway, due to reduced expression of hCAT-1 and hCAT-2B transporters, and membrane depolarization. Inhibition of NO synthesis in cells from IUGR pregnancies was associated with elevated eNOS protein and mRNA levels.

Materials and Methods

IUGR Samples

Umbilical cords were collected after delivery from full-term normal or IUGR pregnancies within 30 weeks (University of Concepcion Ethics Committee approval and patient informed consent were

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TABLE 1. Clinical Characteristics of Patients

<table>
<thead>
<tr>
<th></th>
<th>Normal Pregnancies (n=23)</th>
<th>IUGR Pregnancies (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>24.1±1.5 (21 to 26)</td>
<td>26.4±1.4 (24 to 28)</td>
</tr>
<tr>
<td>Parity</td>
<td>1 (0 to 2)</td>
<td>1 (0 to 2)</td>
</tr>
<tr>
<td>Maternal height, cm</td>
<td>160±101 (145 to 170)</td>
<td>161±98 (142 to 169)</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>38.4±0.2 (38.0 to 39.1)</td>
<td>38.2±0.4 (37.3 to 39.5)</td>
</tr>
<tr>
<td>Fetal sex</td>
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<tr>
<td>Male</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>3540±101 (3280 to 3780)</td>
<td>2571±78 (2199 to 2943)*</td>
</tr>
<tr>
<td>Ponderal index, g/cm²×100</td>
<td>3.11±0.2 (3.08 to 3.15)</td>
<td>2.44±0.21 (2.38 to 2.47)*</td>
</tr>
</tbody>
</table>

IUGR indicates intrauterine growth retardation. Data are expressed as mean±SEM (range) or numbers.

*P<0.05 vs Normal.

obtained). Gestational age was estimated by ultrasonography before the 12th week of pregnancy, and IUGR was defined prospectively before delivery as weight below the fifth centile for gestational age and sex according to the standard Shepard’s weight chart (Table 1). IUGR fetuses had a postnatal confirmation of a birthweight <5th percentile for Chile population standard. All pregnancies were singleton and pregnant women were normotensive, nonsmoking, non-alcoholic or drug consuming, and without intrauterine infection or any other medical or obstetrical complications.

Cell Culture

Endothelium isolated by collagenase (0.25 mg/mL) digestion from human umbilical veins was cultured (37°C, 5% CO2) in medium 199 (M199) containing 5 mmol/L d-glucose, 10% new born calf serum, 10% fetal calf serum, 3.2 mmol/L L-glutamine, 100 μmol/L L-arginine, and 100 U/mL penicillin-streptomycin (primary culture medium). Twenty-four hours before an experiment, the incubation medium was changed to sera-free M199. Experiments were performed in passage 2 confluent cells.

L-Arginine Transport

Cells were rinsed with Krebs solution (in mmol/L): NaCl 131; KCl 3.6; NaHCO3 25; NaH2PO4 1; D-glucose 5; HEPES 20; CaCl2 2.5; MgCl2 1 (pH 7.4, 37°C). Kinetic experiments were performed in cells incubated for 1 minute with Krebs containing L-arginine (7.5 to 1000 μmol/L) and 2 μCi/mL L-[3H]arginine. The effect of the system γ inhibitor N-ethylmaleimide (NEM, 0.1 to 300 μmol/L, 30 minutes) or the NO donor S-nitroso-N-acetyl-L-penicillamine (SNAP, 0.1 to 300 μmol/L, 15 minutes) on transport was assayed. Half-maximal effect of NEM and SNAP were at 12±5 and 23±4 μmol/L, respectively, with maximal effect at 100 μmol/L. This concentration was used in subsequent experiments. In trans-stimulation experiments, cells were incubated (2 hours) with primary culture medium containing 10 mmol/L l-lysine before measuring l-arginine transport (100 μmol/L, 1 minute). Monolayers were rinsed (200 μL/well ice-cold PBS) and radioactivity determined as described. Data were analyzed using the computer program EnzFitter and Ultra Fit (Elsevier, Biosoft).

Tetra[3H]phenylphosphonium (TPP+) Influx and Membrane Potential

Influx of 46 nmol/L [3H]TPP+ (0.5 μCi/mL, 15 to 120 seconds), a membrane potential-sensitive probe, was measured in cells incubated with Krebs containing 100 μmol/L L-arginine and 5.5 or 131 mmol/L KCl, or SNAP (100 μmol/L, 15 minutes) as described for l-arginine transport.

Resting membrane potential (whole-cell patch clamp configuration) was recorded using an EPC-7 amplifier (List Medical, Darmstadt, F.R.G.) as described. Patch pipettes (resistance 4 to 6 MO) contained (in mmol/L) KCl 135, CaCl2 0.2, MgCl2 1.6, HEPES 10, EGTA 2, K-ATP 2.5, Li-GTP 0.2 (pH 7.3). NO-effect was assayed by preincubating cells with SNAP (100 μmol/L, 15 minutes). Membrane potential was measured for at least 1 minute in the continuous presence of SNAP, and only recordings with <0.1-mV variations were considered.

cGMP Determination

Cells were preincubated (30 minutes) in Krebs (37°C) containing 100 μmol/L L-arginine and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (BMX, 0.5 mmol/L), in absence or presence of 100 μmol/L N-nitro-L-arginine methyl ester (L-NAME, 30 minutes). Cells were incubated with 0.1 N HCl (1 mL/well, 4°C, 60 minutes) and 800 μL HCl cell extracts were used for radioimmunoassay of cGMP after acetylation.

L-[3H]Citrulline Assay

Cells were incubated with 100 μmol/L L-[3H]arginine (4 μCi/mL, 30 minutes, 37°C) in the absence or presence of 100 μmol/L L-NAME (30 minutes), and digested in 95% formic acid. A sodium ion form of the cation ion-exchange resin Dowex 50W (50X8-200) was calibrated, and 200-μL digested cells were passed through the column. L-[3H]Citrulline was determined in the H2O eluate.

Determination of Nitrate and Nitrite

Nitrate and nitrite levels were measured by Griess reaction as described. After conversion of nitrate to nitrite and cofactors, 100 μL of medium (collected at 0 or after 24 hours incubation) were mixed with 100 μL Griess reagent (1% sulfanilamide+0.1% naphthylethenediamine in 2% phosphoric acid) and optical density was determined at 540 nm.

Western Blot for eNOS

Cells were lysed and proteins were separated by polyacrylamide gel (8%) electrophoresis, transferred to Immobilon-P polyvinylidene difluoride membranes and probed with a primary polyclonal rabbit anti-eNOS (1:1500) or anti-actin (1:2000). Membranes were washed (×6) in Tris buffer saline Tween (TBST, 50 mmol/L Tris/HCl, 150 mmol/L NaCl, 0.02% v/v Tween 20, pH 7.4), and incubated (1 hour) in TBST/0.2% BSA containing horseradish peroxidase-conjugated goat anti-rabbit antibody. Proteins were detected using enhanced chemiluminescence (ECL) detection reagents and quantitated by densitometry using an Ultrascan XL enhanced laser densitometer (LKB Instruments).

High-Performance Liquid Chromatography

Intracellular L-citrulline and L-arginine in cell extracts were analyzed by high-performance liquid chromatography (HPLC). Cells extracts with methanol (96%, 30 minutes), were exposed to 3 cycles of freeze-thawing, centrifuged (1500 rpm, 2 minutes), and the supernatant evaporated to dryness under a stream of nitrogen gas and
resuspended in 100 μL methanol. Aliquots of samples (20 μL) or standards were injected onto a Hypersil Ultratrasphere ODS-5 m reversed-phase HPLC column (Jones Chromatography) in a Kontron 400 Series gradient HPLC system (Kontron Instruments Ltd). Amino acid concentrations were calculated from the peak areas by reference to the area of the internal standard L-homoserine.\(^{18,19}\)

**Semiquantitative Polymerase Chain Reaction**

Cells were rinsed twice with Krebs solution, and the mRNA was extracted using the Dynabeads technique (Dynal, Norway). The mRNA was reverse transcribed into cDNA using oligo(dT\(_{18}\)) plus random hexamers (10 mer) and M-MLV reverse transcriptase (Promega) for 1 hour at 37°C. Polymerase chain reactions (PCR) were performed in a total volume of 20 μL containing 2 μL 10x PCR buffer, 0.8 μL 50 mmol/L Mg\(^{2+}\), 0.4 μL dNTPs, 13.6 μL RNAase-free H\(_2\)O and 0.2 μL Taq DNA polymerase (Gibco Life Technologies) and sequence-specific oligonucleotide primers for human CAT-1, CAT-2A, CAT-2B, and eNOS (0.5 μmol/L). Samples were incubated for 4 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 57°C, 30 seconds at 72°C, and a final extension for 7 minutes at 72°C. Expression of β-actin was used as a reference value. RT-PCR products were sequenced in both directions by Taq dye terminator cycle sequencing with an automated DNA sequencer 373A (Applied Biosystems).

Oligonucleotide primers were for hCAT-1 (sense) 5'-CCAGTACTTC-CGACGAGTTAGA-3', hCAT-2B (sense) 5'-TATCCCGAGTCTTCTGCT-3', hCAT-2A (antisense) 5'-TGCACAGCACCCGGCAACGCTG-3', hCAT-2B (sense) 5'-CTGCTGTCGAGCAGCAGTGTACT-3', hCAT-2B (antisense) 5'-GAGCAGCTCGAGCTCGAGCTGACTG-3', eNOS (sense) 5'-CCAGCTAGCGAAGTCACC-3', eNOS (antisense) 5'-GTCTCGGGAGCATTAGAGGTCAGTCTAG-3', β-actin (sense) 5'-AAACCCGG-GAGAATGACCCAGATCATCTTT-3', β-actin (antisense) 5'-GTGCTGGGACGCATTAGAGGTCAGTCTAG-3', 3'-cyclic GMP-TME, [Tyrosine-\(^{125}\)I] was from ICN. eNOS antibodies were from Cell Signaling, New England Biolabs (UK), and actin antibodies from Santa Cruz Biotechnology.

**Materials**

Newborn and fetal calf serum, agarsore, and buffers were from Gibco Life Technologies. Collagenase Type II (Clostridium histolyticum) was from Boehringer Mannheim and Bradford protein reagent from BioRad Laboratories. Ethidium bromide and Dowex (50WX8-400) was from Boehringer Mannheim and Bradford protein reagent from BioRad Laboratories.

**Statistics**

Values are mean±SEM, where n indicates the number of different cell cultures with 4 to 8 replicate measurements per experiment. Statistical analyses were carried out on raw data using the Peritz\(^{23}\) F multiple means comparison test. A Student’s t test was applied for unpaired data, and \(P<0.05\) was considered statistically significant.

**Results**

**L-Arginine Transport**

Overall rates of L-arginine transport in cells from normal or IUGR pregnancies were fitted best by a Michaelis-Menten equation plus a linear nonsaturable component (Figure 1A), confirming previous observations in HUVECs.\(^{6,16,19}\) Although the \(K_v\) value (nonsaturable component) was lower in cells from IUGR compared with normal pregnancies (Table 2), we could not fit the data with a transport rate equation for two saturable systems acting in parallel. Saturable L-arginine transport (Figure 1B) showed a reduction in the \(V_{\text{max}}\) with no significant changes in apparent \(K_v\) in IUGR compared with normal pregnancies (Table 2). Eadie-Hofstee analyses of transport were linear (Figure 1C), suggesting the presence of a single high-affinity transport site for L-arginine in both cell types. The \(V_{\text{max}}\) was significantly increased by SNAP in cells...
from normal pregnancies, with no significant changes in apparent $K_m$ (Table 2). IUGR-associated reduction of $V_{\text{max}}$ was reversed by SNAP to values in cells from normal pregnancies incubated with this molecule, with no significant changes in apparent $K_m$ (Table 2).

### Cationic Amino Acid Transporter (CAT) Expression

RT-PCR experiments show that HUVECs express hCAT-1 and hCAT-2B. IUGR was associated with reduced hCAT-1 (69±7%, Figure 2A) and hCAT-2B (71±8%, Figure 2B) mRNA levels compared with cells from normal pregnancies. hCAT-2A mRNA was undetectable in our PCR experiments.

### Effect of N-Ethylmaleimide (NEM) and l-Lysine on l-Arginine Transport

l-Arginine (100 μmol/L) transport was inhibited by NEM in cells from normal pregnancies, but IUGR-associated reduction of l-arginine transport was unaltered by NEM (Figure 3A). In cells preincubated for 2 hours with 10 mmol/L l-lysine, basal influx of l-arginine was increased 7.8- and 9.9-fold for normal and IUGR pregnancies, respectively (Figure 3B).

### Membrane Potential

IUGR cells exhibit reduced TPP$^+$ influx compared with cells from normal pregnancies (Figure 3C). TPP$^+$ influx was also inhibited by depolarization with elevated extracellular KCl, and patch-clamp experiments demonstrated that cells from IUGR exhibited membrane depolarization compared with normal pregnancies (Table 3). Changes in membrane potential and TPP$^+$ influx were paralleled by significant reduction of l-arginine transport. Table 3 also shows that SNAP increased l-arginine transport and [3H]TPP$^+$ influx, and induced membrane hyperpolarization in cells from normal pregnancies. IUGR-associated reduction of l-arginine transport and [3H]TPP$^+$ influx and membrane depolarization were reversed by SNAP to values in cells from normal pregnancies in the presence of SNAP.

### eNOS Activity and Expression

Lower l-[3H]citrulline formation from l-[3H]arginine (Figure 4A) and intracellular cGMP levels (Figure 4B) were observed in cells from IUGR compared with normal pregnancies. l-[3H]Citrulline formation and intracellular cGMP were significantly reduced by L-NAME (inhibitor of eNOS), in cells from normal pregnancies. However, L-NAME did not alter IUGR-associated reduction of l-[3H]citrulline formation and intracellular cGMP. IUGR was also associated with lower levels of nitrite (0.13±0.04 μmol/L, n=6; $P<0.05$) compared with cells from normal pregnancies (0.41±0.11 μmol/L). Parallel experiments demonstrated that IUGR was associated with higher ($P<0.05$) eNOS protein (1.7±0.2 fold) and eNOS mRNA (1.9±0.2 fold) levels compared with normal pregnancies (Figure 5).

### Plasma and Intracellular l-Arginine and l-Citrulline

l-Arginine concentration in cells from IUGR pregnancies was lower (0.4±0.2 mmol/L, n=5; $P<0.05$) compared with cells from normal pregnancies (1.5±0.3 mmol/L, n=5). In addition, intracellular l-citrulline in IUGR cells was also low (0.13±0.06 mmol/L, n=4; $P<0.05$) compared with cells from

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**TABLE 2. Effect of IUGR and NO on l-Arginine Transport Kinetics in Human Umbilical Vein Endothelial Cells**

<table>
<thead>
<tr>
<th></th>
<th>$K_m$, μmol/L</th>
<th>$V_{\text{max}}$, pmol (μg protein)$^{-1}$ min$^{-1}$</th>
<th>$V_{\text{max}}/K_m$, pmol (μg protein)$^{-1}$ min$^{-1}$ μmol/L$^{-1}$</th>
<th>$K_s$, pmol (μg protein)$^{-1}$ min$^{-1}$ μmol/L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>159±15</td>
<td>5.8±0.2</td>
<td>0.036±0.001</td>
<td>0.031±0.001</td>
</tr>
<tr>
<td>SNAP</td>
<td>132±18</td>
<td>9.9±0.4*</td>
<td>0.075±0.006*</td>
<td>0.030±0.001</td>
</tr>
<tr>
<td>IUGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>137±14</td>
<td>3.3±0.1*</td>
<td>0.024±0.001*</td>
<td>0.025±0.001*</td>
</tr>
<tr>
<td>SNAP</td>
<td>117±21</td>
<td>7.2±0.5*</td>
<td>0.062±0.008†</td>
<td>0.032±0.001†</td>
</tr>
</tbody>
</table>

Kinetics for saturable l-arginine transport in endothelial cells from normal (Normal) or intrauterine growth retardation (IUGR) pregnancies exposed to Krebs solution (Control) or Krebs containing S-nitroso-N-acetyl-l-cysteine (SNAP, 100 μmol/L, 15 minutes) (see Materials and Methods). Values are mean±SEM, n=9.

*P<0.05 vs Control in Normal; †P<0.05 vs Control in IUGR.

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**Figure 2.** Effect of IUGR on the cationic amino acid transporter (CAT) mRNA levels in human umbilical vein endothelial cells. mRNA was extracted from cells isolated from normal (Control) or IUGR pregnancies (see Materials and Methods). mRNA was reverse transcribed into cDNA and PCR were performed using sequence-specific oligonucleotide primers for (A) human CAT-1 (449 bp) or (B) CAT-2B (357 bp). β-Actin (350 bp) was used as housekeeper. Data are representative of similar results in cell cultures from normal (n=5) or IUGR (n=5) pregnancies.
Figure 3. N-Ethylmaleimide and L-lysine effect on saturable L-arginine transport and effect of IUGR on tetraphenylphosphonium (TPP⁺) influx in human umbilical vein endothelial cells. Cells were isolated from normal (open bars, n=4) or IUGR (filled bars, n=4) pregnancies, and L-arginine transport (100 μmol/L, 1 minute, 37°C) was measured in cells preincubated for 2 hours in medium 199 (Control) or medium 199 containing (A) 100 μmol/L N-ethylmaleimide (NEM) or (B) 10 mmol/L L-lysine. C, Cells from normal (c, n=12) or IUGR (e, n=11) pregnancies were exposed to Krebs solution (37°C) containing 46 nmol/L tetra[3H]phenylphosphonium ([3H]TPP⁺, 0.5 μCi/mL) for the specified periods of time (see Materials and Methods). Values are mean±SEM; *P<0.04 vs all other values.

Discussion

This study shows that human umbilical vein endothelial cells (HUVECs) isolated from fetuses with intrauterine growth retardation (IUGR) exhibit reduced L-arginine transport associated with a lower $V_{\text{max}}$ and expression of the high-affinity membrane transport systems $y′$/hCAT-1 and $y′$/hCAT-2B. Inhibition of L-arginine transport is paralleled by membrane depolarization and reduced synthesis of NO. The inhibition of L-arginine transport and changes in membrane potential induced by IUGR are reversed by external NO, but cells from IUGR pregnancies show increased eNOS mRNA and protein levels, which could be an adaptive response to the reduced NO levels and L-arginine transport exhibited by endothelial cells from fetuses with IUGR.

L-arginine transport in HUVECs is mediated by the high-affinity systems $y′$/CAT-1 ($K_m \approx 100$ to 200 μmol/L) and $y′$/CAT-2B ($K_m \approx 200$ to 400 μmol/L), with the first likely predominating at physiological concentrations of extracellular L-arginine. HUVECs from fetuses with IUGR exhibit a reduced L-arginine transport associated with lower $V_{\text{max}}$, with no significant changes in the apparent $K_m$, compared with L-arginine transport in cells from normal pregnancies. A report shows that endothelial cells from fetuses with IUGR exhibit reduced uptake of 250 μmol/L L-arginine, but further characterization of transport was not performed. Our results show that the apparent $K_m$ estimated for L-arginine transport in IUGR ($K_m = 137$ μmol/L) is within the range of values reported for L-arginine transport in this cell type from normal pregnancies, suggesting that IUGR-associated reduction of L-arginine transport is not due to changes in the affinity of L-arginine transporters in HUVECs.

Exogenous NO (from SNAP) increased TPP⁺ influx and L-arginine transport and induced membrane hyperpolarization in cells from IUGR or normal pregnancies. It has been reported that SNAP increases L-arginine transport in bovine aortic endothelium, which could support the hypothesis that NO acutely modulates L-arginine transport by a mechanism involving membrane hyperpolarization in IUGR endothelium. Furthermore, because SNAP-induced increase of L-arginine transport in IUGR cells was also associated with increased $V_{\text{max}}$, with no significant changes in the apparent $K_m$, NO-mediated increase of L-arginine transport could be due to a higher transport activity ($V_{\text{max}}/K_m$, Table 2), rather than alterations in the affinity of L-arginine transporters.

Recent studies have reported expression and activity of the very high-affinity transport system $y^\prime$L, which exchanges L-leucine by L-arginine in a Na⁺-dependent manner, with apparent $K_m$ between 1 μmol/L and 40 μmol/L in HUVECs. Because the apparent $K_m$ for L-arginine transport is ~140 μmol/L in our study, it is unlikely that system $y^\prime$L would contribute significantly to overall L-arginine transport. This is supported by our findings showing that L-arginine transport (100 μmol/L) is reduced by 93% by NEM, a competitive inhibitor of system $y^\prime$, in HUVECs from normal pregnancies. Because L-arginine transport rates in cells from IUGR pregnancies are similar to rates in cells from normal pregnancies treated with NEM, and L-arginine transport was not further altered by this inhibitor in IUGR cells, it...
is feasible that IUGR-associated reduction of transport is associated with changes in the activity of system y\(^\text{CATs}\) rather than system y\(^{	ext{L}}\). This is also supported by results showing that IUGR-associated inhibition of l-arginine transport was not due to changes in the apparent \(K_m\) (Table 2).

l-Arginine transport was trans-stimulated (9.9-fold) by the cationic amino acid l-lysine in cells from IUGR pregnancies. As l-arginine transport is trans-stimulated by 9.8-fold or 1.8-fold in Xenopus oocytes injected with hCAT-1 or hCAT-2B mRNA, respectively,\(^{32}\) we could suggest that hCAT-1, rather than hCAT-2B transporters, are involved in l-arginine transport in cells from IUGR pregnancies. PCR experiments revealed expression of the high-affinity hCAT-1 and hCAT-2B transporters, but not the low-affinity hCAT-2A transporter \((K_m \approx 2 \text{ mmol/L})\) or a splice variant of hCAT-2B\(^{26,27}\) in endothelial cells from IUGR pregnancies. IUGR was associated with reduced hCAT-1 and hCAT-2B mRNA levels, raising the possibility that IUGR-associated reduction of l-arginine transport could be due to lower expression of hCAT-1, hCAT-2B or both transporters in HUVECs.

IUGR is associated with elevated maternal arterial plasma level of l-arginine,\(^{31}\) and a significant reduction in the intracellular l-arginine concentration was detected in HUVECs from IUGR compared with normal pregnancies. A possible explanation for this finding is that IUGR-associated reduction of intracellular l-arginine could be due to the reduced l-arginine transport exhibited by IUGR endothelium. Supplementation with l-arginine significantly prevents fetal growth retardation in animal models of IUGR,\(^{12,13}\) suggesting and reinforcing the critical role of l-arginine availability, and possibly l-arginine transport, for fetal development and growth.\(^{13,25,34}\)

IUGR was associated with reduced synthesis of l-citrulline from l-arginine, reduced levels of nitrite, intracellular cGMP, l-citrulline, and l-arginine, suggesting that NO synthesis is impaired in cells from fetuses with this pathology. The functional role of NO in normal fetal growth has been demonstrated in several studies where inhibition or deletion of endothelial NO synthase (eNOS) is associated with increased frequency of IUGR pregnancies.\(^{9,10,13,35–39}\) Long-term inhibition of endothelium-derived NO induces IUGR, which is reversed by treatment with l-arginine in rats,\(^{13}\) supporting the possibility that availability and uptake of l-arginine by endothelium are necessary for preventing abnormal growth of fetuses.\(^{13,25,34}\) IUGR induced by lack of NO may also result from a reduced amino acid delivery, including l-arginine, to the fetus,\(^{38}\) which could complement our observations of reduced intracellular l-arginine and l-citrulline in HUVECs. This is supported by recent studies in genetically-engineered CAT2-deficient mice where system y\(^{L}\)-mediated l-arginine transport is strongly reduced, and NO synthesis via iNOS requires extracellular l-arginine uptake.\(^{40}\) As mentioned above, in our study the NO donor SNAP increased l-arginine transport as reported to occur in other endothelial cells.\(^{30}\) NO-induced hyperpolarization of endothelial cells is in part due to a direct action of NO on K\(^+\) channels.\(^{41}\) IUGR-associated inhibition of l-arginine transport and TPP\(^{+}\) influx are associated with membrane depolarization in HUVECs. These changes are reversed by SNAP, suggesting that NO could change ion channel activity (and hence l-arginine transport) in this cell type. Thus, reduced NO level may in part explain the reduced l-arginine transport exhibited by IUGR cells.

Higher eNOS mRNA and protein levels were detected in cells from IUGR compared with normal pregnancies. A previous report has shown that NO inhibited eNOS activity, but did not alter mRNA or protein levels for eNOS in lamb fetal main pulmonary artery endothelium.\(^{42}\) However, incubation of lamb fetal intrapulmonary artery endothelium with L-NAME decreased, and exogenous NO increased, eNOS activity, protein, and mRNA levels.\(^{43}\) Despite the high L-NAME concentration used in the latter study (2 mmol/L), these contradictory findings could result from the different cell types studied. Our results of higher eNOS mRNA and

<table>
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<th></th>
<th>l-Arginine Transport, pmol ((\mu)g protein(^{-1}) min(^{-1}))</th>
<th>[(^3)H]TPP(^+) Influx, pmol ((\mu)g protein(^{-1}) min(^{-1}))</th>
<th>(E_m), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.4*</td>
<td>1.7 ± 0.2*</td>
<td>−65.1 ± 0.4*</td>
</tr>
<tr>
<td>KCl (131 mmol/L)</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>−25.4 ± 0.7</td>
</tr>
<tr>
<td>SNAP</td>
<td>9.5 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>−75.3 ± 0.2</td>
</tr>
<tr>
<td>IUGR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>−37.2 ± 3.0</td>
</tr>
<tr>
<td>KCl (131 mmol/L)</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.2</td>
<td>−15.3 ± 2.1</td>
</tr>
<tr>
<td>SNAP</td>
<td>5.8 ± 0.7†</td>
<td>2.9 ± 0.2†</td>
<td>−75.2 ± 0.3†</td>
</tr>
</tbody>
</table>

\(\frac{P<0.05}{\text{vs}}\) values in corresponding columns for l-arginine transport, [\(^3\)H]TPP\(^+\) influx, and \(E_m\); † P<0.05 vs Control and 131 mmol/L KCl in IUGR.
protein and lower eNOS activity could represent an adaptive response of HUVECs from IUGR pregnancies to a reduced NO level. In bovine main pulmonary artery endothelium, eNOS expression is increased by exogenous cGMP, and a cGMP-dependent positive-feedback control of eNOS has been proposed. Our results show that increased eNOS expression was paralleled by reduced intracellular cGMP levels. We hypothesize that eNOS gene expression could be regulated by a negative-, rather than a positive-, feedback control mechanism in HUVECs. Inhibition of eNOS activity by NO could also be due to a direct action of NO on eNOS or increased levels of superoxide anion. This possibility is unlikely in HUVECs because incubation of cells with superoxide dismutase, a scavenger for superoxide anion, did not alter IUGR-associated changes in eNOS expression and NO synthesis (not shown). The disparity between results in HUVECs and those reported in ovine fetal pulmonary endothelium could well be due to differences in cell source (pulmonary arteries versus umbilical veins) and species (human versus lamb).

Figure 4. Effect of IUGR on L-citrulline formation and intracellular cGMP level in human umbilical vein endothelial cells. Cells were isolated from normal (open bars, \( n = 6 \)) or IUGR (filled bars, \( n = 6 \)) pregnancies, deprived of serum for 24 hours, washed, and preincubated for 30 minutes with Krebs solution in the absence (Control) or in the presence of \( \mathrm{N}^- \)-nitro-L-arginine methyl ester (L-NAME, 30 minutes), and (A) intracellular cGMP or (B) L-[\( ^3 \)H]citrulline formation from L-[\( ^3 \)H]arginine (4 \( \mu \)Ci/mL, 30 minutes, 37°C) were determined (see Materials and Methods). Values are mean\( \pm \)SEM; \( * P < 0.05 \) vs all other values.

Figure 5. Effect of IUGR on eNOS protein and mRNA levels in human umbilical vein endothelial cells. Total protein and mRNA were extracted from cells isolated from normal (Control) or IUGR pregnancies (see Materials and Methods). A, Endothelial cells were deprived of serum for 24 hours, washed, and Western blotted for total eNOS or actin as housekeeper. Data are representative of similar blots obtained in 9 different cell cultures. B, mRNA was reversed transcribed into cDNA, and PCR was performed using sequence-specific oligonucleotide primers for human eNOS (350 bp) or \( \beta \)-actin (350 bp) as housekeeper. Data are representative of similar results in cell cultures from normal (\( n = 5 \)) or IUGR (\( n = 5 \)) pregnancies.

This study has demonstrated that endothelial cells from fetuses with IUGR exhibit an altered \( \ell \)-arginine/NO pathway, which is associated with reduced \( \ell \)-arginine transport and NO synthesis. Inhibition of \( \ell \)-arginine transport could be due to lower expression of hCAT-1 and hCAT-2B transporters, membrane depolarization, or reduced activity of eNOS. This is the first direct demonstration of a reduced expression of hCAT-1 and hCAT-2B mRNA in human umbilical vein endothelium from IUGR fetuses. Because several studies have demonstrated that either \( \ell \)-arginine availability or eNOS activity are determinant in the normal fetal growth, we propose that reduced eNOS activity and \( \ell \)-arginine transport may be crucial physiological processes involved in the etiology of IUGR in human pregnancies.

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References


Intrauterine Growth Retardation Is Associated With Reduced Activity and Expression of the Cationic Amino Acid Transport Systems $y^+$/hCAT-1 and $y^+/hCAT-2B$ and Lower Activity of Nitric Oxide Synthase in Human Umbilical Vein Endothelial Cells

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