Rapid Stimulation of L-Arginine Transport by D-Glucose Involves p42/44\textsuperscript{mapk} and Nitric Oxide in Human Umbilical Vein Endothelium

Carlos Flores, Susana Rojas, Claudio Aguayo, Jorge Parodi, Giovanni Mann, Jeremy D. Pearson, Paola Casanello, Luis Sobrevia

Abstract—D-Glucose infusion and gestational diabetes induce vasodilatation in humans and increase l-arginine transport and nitric oxide (NO) synthesis in human umbilical vein endothelial cells. High D-glucose (25 mmol/L, 2 minutes) induced membrane hyperpolarization and an increase of l-arginine transport (V\textsubscript{max} 6.1±0.7 versus 4.4±0.1 pmol/µg protein per minute) with no change in transport affinity (K\textsubscript{m} 105±9 versus 111±16 µmol/L). L-[\textsuperscript{3}H]Citrulline formation and intracellular cGMP, but not intracellular Ca\textsuperscript{2+}, were increased by high D-glucose. The effects of D-glucose were mimicked by levcromakalim (ATP-sensitive K\textsuperscript{+} channel blocker), paralleled by p42/p44\textsuperscript{mapk} and Ser\textsuperscript{1177}—endothelial NO synthase phosphorylation, inhibited by N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME; NO synthesis inhibitor), glibenclamide (ATP-sensitive K\textsuperscript{+} channel blocker), KT-5823 (protein kinase G inhibitor), PD-98059 (mitogen-activated protein kinase kinase 1/2 inhibitor), and wortmannin (phosphatidylinositol 3-kinase inhibitor), but they were unaffected by calphostin C (protein kinase C inhibitor). Elevated D-glucose did not alter superoxide dismutase activity. Our findings demonstrate that the human fetal endothelial L-arginine/NO signaling pathway is rapidly activated by elevated D-glucose via NO and p42/44\textsuperscript{mapk}. This could be determinant in pathologies in which rapid fluctuations of plasma D-glucose may occur and may underlie the reported vasodilatation in early stages of diabetes mellitus. (Circ Res. 2003;92:64-72.)

Key Words: humans ■ endothelium ■ glucose ■ arginine ■ nitric oxide

The cationic amino acid l-arginine is the substrate for nitric oxide (NO) synthesis via endothelial NO synthase (eNOS)\textsuperscript{1} and is taken up primarily by the Na\textsuperscript{+}-independent high-affinity (K\textsubscript{m} =100 to 400 µmol/L) systems y\textsuperscript{+}/CAT-1 and y\textsuperscript{+}/CAT-2B (where CAT indicates cationic amino acid transporter) in human umbilical vein endothelial cells (HUVECs).\textsuperscript{2,3} L-Arginine transport and NO synthesis (l-arginine/NO pathway) are increased in HUVECs from patients with gestational diabetes.\textsuperscript{2} Interestingly, long-term incubation (24 hours) of HUVECs from normal pregnancies with elevated D-glucose mimics the effect of gestational diabetes on the L-arginine/NO pathway.\textsuperscript{4} In addition, elevated D-glucose for 24 hours\textsuperscript{5-5} or 5 days\textsuperscript{6} increases eNOS gene expression. A recent report shows that D-glucose infusion induces vasodilatation in humans,\textsuperscript{7} and in animal models, an elevation of plasma D-glucose results in rapid (seconds to minutes) vasodilatation.\textsuperscript{9-10} Therefore, rapid fluctuations in the D-glucose level are crucial in maintaining human fetal endothelial function.\textsuperscript{2-5,11}

D-Glucose activates protein kinase C (PKC), an enzyme involved with long-term stimulation of the L-arginine/NO pathway,\textsuperscript{5,12-14} and (within 1 hour) p42 and p44 mitogen-activated protein (MAP) kinases (p42/44\textsuperscript{mapk}).\textsuperscript{5,14,15} p42/44\textsuperscript{mapk} activation may itself be dependent on PKC activation and NO synthesis.\textsuperscript{5,14} However, the effect of short-term incubation with elevated D-glucose on the endothelial L-arginine/NO pathway has not been investigated.\textsuperscript{4,11,16,17}

The present study shows that a 2-minute incubation with 25 mmol/L D-glucose increases L-arginine transport and NO synthesis in HUVECs. The underlying cellular mechanisms involve phosphorylation of eNOS at Ser\textsuperscript{1177} via phosphatidylinositol 3-kinase (PI3-k) and activation of eNOS and p42/p44\textsuperscript{mapk} by D-glucose.\textsuperscript{18}

Materials and Methods

Cell Culture

Human umbilical vein endothelium was isolated (collagenase digestion 0.25 mg/mL) and cultured (37°C, 5% CO\textsubscript{2}, confluent passage 2) in medium 199 containing 5 mmol/L D-glucose, 10% newborn 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).\textsuperscript{2-4} Before an experiment (24 hours), the incubation medium was changed to serum-free medium 199.
1-Arginine Transport
1-Arginine transport (1 μCi/mL, 37°C, 1 minute) was determined in cells preincubated (15 seconds to 5 minutes) with Krebs solution (mmol/L: NaCl 131, KCl 5.6, NaHCO3, 25, Na2HPO4, 1, HEPES 20, CaCl2, 2.5, and MgCl2, 1 [pH 7.4, 37°C] containing 5 or 25 mmol/L D-glucose, 25 mmol/L L-glucose, or 5 mmol/L D-glucose plus 20 mmol/L d-mannitol (osmotic controls).2-4 1-Arginine transport was also determined in Krebs solution in which NaCl was replaced by equimolar concentrations of choline chloride2-4 or in cells incubated (30 minutes) with KCl (5.5 to 131 mmol/L), with NaCl decreased equivalently, or with 131 mmol/L KCl for 2, 4, 10, 20, or 30 minutes. In trans-stimulation experiments, cells were preincubated (2 hours) with primary culture medium containing 10 mmol/L L-lysine. Cell-associated radioactivity and data analyses were performed as described.2-4

Intracellular Ca2+
Cells on glass coverslips were loaded (30 minutes, 23°C) with the acetoxyethyl derivative of fluo 3 (5 μmol/L). Coverslips were transferred to an experimental bath with Krebs solution containing 5 or 25 mmol/L D-glucose, and Ca2+ was imaged using a Zeiss LSM 410 confocal microscope.4

Western Blots
After pretreatment with 10 μmol/L PD-98059 (30 minutes), 100 μmol/L SNAP (2 to 5 minutes), or 30 mmol/L wortmannin (30 minutes), the cells were incubated with 5 or 25 mmol/L D-glucose (2 minutes). Cell protein extracts were probed with a primary polyclonal mouse antiphosphorylated (1:1000) or nonphosphorylated (1:1500) p44/p42MAPK, rabbit anti-eNOS (1:2500) or anti-phosphorylated Ser1177-eNOS (1:2500) antibodies, and horseradish peroxidase-conjugated goat secondary antibodies as described.3,5 Primary polyclonal mouse anti-actin (1:2000) served as the internal control. Proteins were detected by enhanced chemiluminescence and quantified by densitometry (Ultrorcan XL enhanced laser densitometer, LKB Instruments).3,5

Semiquantitative PCR
Extracted mRNA (Dynal) was reversed-transcribed into cDNA using oligo(dT)18 plus random hexamers (10-mer) and M-MLV reverse transcriptase (Promega) for 1 hour at 37°C.1 Polymerase chain reactions (PCRs) were performed in 20-μL samples (2 μL of 10× PCR buffer, 0.8 μL of 50 mmol/L MgCl2, 0.4 μL dNTPs, 13.6 μL RNA-free H2O, 0.2 μL Taq DNA polymerase, and 0.5 μmol/L sequence-specific oligonucleotide primers for human CAT-1, CAT-2A, or CAT-2B). Samples were incubated (4 minutes, 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. β-Actin expression was used as a reference value. Reverse transcription (RT)-PCR products were sequenced in both directions by T7u dioxy terminator cycle sequencing (automated DNA sequencer 373A, Applied Biosystems).1 Oligonucleotide primers were as follows: hCAT-1 (sense) 5'-CCAGTACTTCCCCAGCAAGTTAGA-3', hCAT-1 (antisense) 5'-CATCCCAACAGCAAAACCCGACC-3', hCAT-2A (sense) 5'-TATCCCCATTTTTTTGCTGTGTC-3', hCAT-2A (antisense) 5'-TOCACTCAACCTGTGACCAACT-3', hCAT-2B (sense) 5'-TCCCATGCTCGTGTAATCTCATA-3', hCAT-2B (antisense) 5'-GCTGCTGTAATCTCATA-3', β-actin (sense) 5'-AACCAGGAGAATGACAGATCATCTTCT-3', and β-actin (antisense) 5'-AGCAGCCTGTCGCGGATGTCCATTCG-3'. Expected size products were as follows: hCAT-1, 450 bp; hCAT-2A, 690 bp; hCAT-2B, 360 bp; and β-actin, 350 bp.

SOD Activity and α-Tocopherol Experiments
Cells were homogenized in buffer containing 50 mmol/L Tris-(hydroxymethyl)-ammonium, 100 mmol/L potassium chloride, 0.02% Triton X-100, 100 mmol/L sodium pyrophosphate, and 100 mmol/L sodium fluoride (pH 7.4), which was supplemented with trypsin inhibitors (4 mg/mL aprotinin, 1 mg/mL benzamidine, 5 μg/mL leupeptin, and 200 μmol/L sodium orthovanadate). Aliquots (1 mg protein/mL) were incubated (25°C, 2 minutes) with potassium phosphate buffer (50 mmol/L, pH 10.2) containing adrenochrome (200 μmol/L) and epinephrine (10 μmol/L), and absorbance was measured at 480 nm. Superoxide dismutase (SOD) activity was calculated from the inhibition curve for epinephrine auto-oxidation versus protein concentration. Basal absorbance (100% activity) was the reaction in the absence of cell extracts.26 Cells were also preincubated (30 minutes) with α-tocopherol (500 μg/mL, 1% ethanol).27

Materials
Sera, agarose, and buffers were from GIBCO Life Technologies. Collagenase type II (Clostridium histolyticum) was from Boehringer-Mannheim, and Bradford protein reagent was from Bio-Rad Laboratories. LNAME and SNAP were from Calbiochem. Ethidium
bromide, Dowex (50WX8-400), and all other reagents were from Sigma Chemical Co. L-[2,3-3H]Arginine (36.1 Ci/mmol), d-[1,4C]mannitol (49.3 mCi/mmol), [γ-32P]ATP, and [3H]TPP’ (37 Ci/mmol) were from NEN. 3’-5’cGMP-TME was from ICN. Antibodies were from Cell Signaling, New England Biolabs.

Statistical Analysis
Values are mean±SEM, where n indicates the number of different cell cultures (4 to 8 replicates per experiment). Statistical analyses were carried out on raw data using the Peritz F multiple-means comparison test. A Student t test was applied for unpaired data, and a value of P<0.05 was considered statistically significant.

Results

L-Arginine Transport
Elevated d-glucose (2 minutes), but not L-glucose or D-mannitol, stimulated L-arginine transport (half-maximal effect [K1/2] 13±2 mmol/L d-glucose) (Figure 1A). Basal transport rates increased significantly after 30 seconds of exposure to elevated d-glucose (K1/2 25±5 seconds), with maximal rates achieved within 1 minute and sustained over 5 minutes (Figure 1B). Subsequent experiments were performed using 25 mmol/L d-glucose for 2 minutes. d-Glucose-stimulated L-arginine transport decreased to basal values within 5 minutes after reexposure of cells to 5 mmol/L d-glucose (Figure 1B). RT-PCR analysis detected only hCAT-1 and hCAT-2B mRNA in HUVECs (Figure 1C).

Elevated d-glucose had no effect on the nonsaturable component (K0) of overall L-arginine transport but increased Vmax with no change in apparent Km (Figure 2A, Table 1).

Cell incubation with L-lysine (10 mmol/L, 2 hours) increased 6.5-fold the L-arginine transport in 5 mmol/L d-glucose (Figure 2B). However, L-arginine transport was increased 2.9-fold in cells exposed to 25 mmol/L d-glucose (last 2 minutes of the 2-hour incubation with L-lysine) (Figure 2B). d-Glucose stimulation and trans-stimulation by L-lysine of L-arginine transport was unaltered in Na+-free Krebs solution (not shown).

TPP+ Influx and Membrane Potential
Elevated d-glucose increased TPP+ influx (1.8-fold) and caused membrane hyperpolarization (Table 2). TPP+ influx and L-arginine transport were inhibited by KCl-induced membrane depolarization. Glibenclamide (K+ATP channel blocker) blocked d-glucose–increased L-arginine transport and changes in TPP+ influx and Em (Table 2), Levromakalim (K+ATP channel activator) hyperpolarized the plasma membrane and increased L-arginine transport and TPP+ influx only in 5 mmol/L d-glucose; effects were blocked by glibenclamide (Table 2). The effects of d-glucose were also blocked by wortmannin (not shown). Preloading cells with L-lysine did not alter Em (−66±1.3 mV, P>0.05; n=29 cells) compared with control cells (−67.5±0.5 mV, P>0.05; n=45 cells). L-Arginine transport was inhibited by KCl with half-maximal inhibition at 12±2 and 17±4 mmol/L KCl for 5 and 25 mmol/L d-glucose, respectively. The time needed to induce half-maximal inhibition of transport with 131 mmol/L KCl was similar in cells in 5 mmol/L d-glucose (6.5±0.6 minutes) compared with 25 mmol/L d-glucose (7.2±0.6 minutes).

Figure 1. Effect of d-glucose on L-arginine transport and CAT expression. A, L-Arginine transport (100 μmol/L, 1 minute, 37°C) in HUVECs incubated (2 minutes) with increasing concentrations of d-glucose (○), L-glucose (□), or 5 mmol/L d-glucose plus 5 to 20 mmol/L d-mannitol (.), *P<0.05 and **P<0.04 vs 5 or 7.5 mmol/L d-glucose and corresponding values in L-glucose and d-glucose+d-mannitol. B, Time course of effect of d-glucose on L-arginine transport (as in panel A) in cells incubated for 0 to 5 minutes in 5 mmol/L d-glucose (○), 25 mmol/L d-glucose (○), or 5 mmol/L d-glucose +20 mmol/L d-mannitol (□). High d-glucose–containing Krebs solution was replaced by 5 mmol/L d-glucose, and cells were incubated for 10 minutes. *P<0.04 vs all other values. Values are mean±SEM (n=13). C, RT-PCR for mRNA from cells in 5 mmol/L d-glucose. mRNA was reversed-transcribed into cDNA, and PCR was performed for human CAT-1 (lane 2, 449 bp), CAT-2A (lane 3, 690 bp), or CAT-2B (lane 4, 357 bp). Lane 5 is β-actin (350 bp), and lane 1 is DNA ladder (100 to 2000 bp). Data are representative of 14 cell cultures.
l-arginine transport, L-NAME also inhibited the effect of D-glucose on L-arginine transport $V_{max}$ (Table 1), TPP$^+$ influx, and $E_m$ (Table 2); however, L-NAME did not alter TPP$^+$ influx or $E_m$ in 5 mmol/L D-glucose. Other experiments show that SNAP (NO donor) induces TPP$^+$ influx, l-arginine transport, and membrane hyperpolarization only in 5 mmol/L D-glucose (Table 2) and that dibutylryl cGMP (dbcGMP) increased l-arginine transport (Figure 4A) and TPP$^+$ influx (Figure 4B). The effects of D-glucose and dbcGMP were blocked by KT-5823, a PKG inhibitor (Table 2).

PKC and MAP Kinase Involvement
PKC activity was unaltered at up to 5 minutes of incubation with high D-glucose (Figure 5A) or after the addition of SNAP (not shown). Furthermore, calphostin C (PKC inhibitor) had no effect on D-glucose–increased l-arginine transport (Figure 5B), TPP$^+$ influx, or NO synthesis (not shown). However, longer incubation with elevated D-glucose (>10 minutes) increased membrane PKC activity (not shown), confirming our previous observations in HUVECs. In contrast, high D-glucose induced p42/p44$^{mek}$ phosphorylation (Figure 6A), an effect blocked by PD-98059 and mimicked by brief exposure (2 minutes) to SNAP (Figure 6B). Interestingly, the effect of D-glucose on p42/p44$^{mek}$ phosphorylation was blocked by wortmannin (Figure 6C). PD-98059 also blocked the stimulatory effects of elevated D-glucose and SNAP on TPP$^+$ influx, l-arginine transport, and $E_m$ (Table 2).

SOD Activity and α-Tocopherol Effect
SOD activity in cells in 5 mmol/L D-glucose (5.5 ± 0.6 U/mL) was not significantly altered ($P > 0.05, n = 5$) by 25 mmol/L D-glucose (6.1 ± 1 U/mL). Extracellular SOD or α-tocopherol did not block ($P > 0.05, n = 4$ to 8) the effect of D-glucose on l-arginine transport (5.6 ± 0.3 and 5.8 ± 0.6 pmol/μg protein per minute, respectively), l-citrulline formation (3.1 ± 0.2 and

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**TABLE 1. D-Glucose Effect on L-Arginine Transport in HUVECs**

<table>
<thead>
<tr>
<th>D-Glucose</th>
<th>$K_p$, μmol/L</th>
<th>$V_{max}$, pmol/μg Protein per Minute</th>
<th>$V_{max}/K_p$, pmol/μg Protein per Minute per μmol/L</th>
<th>$K_p$, pmol/μg Protein per Minute per μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mmol/L</td>
<td>105 ± 9</td>
<td>4.4 ± 0.1</td>
<td>0.044 ± 0.011</td>
<td>0.002 ± 0.0007</td>
</tr>
<tr>
<td>PD-98059</td>
<td>123 ± 12</td>
<td>4.2 ± 0.2</td>
<td>0.034 ± 0.012</td>
<td>0.003 ± 0.0012</td>
</tr>
<tr>
<td>L-NAME</td>
<td>97 ± 16</td>
<td>3.8 ± 0.3</td>
<td>0.039 ± 0.016</td>
<td>0.003 ± 0.0009</td>
</tr>
<tr>
<td>25 mmol/L</td>
<td>111 ± 16</td>
<td>6.1 ± 0.7†</td>
<td>0.055 ± 0.012†</td>
<td>0.003 ± 0.0011</td>
</tr>
<tr>
<td>PD-98059</td>
<td>133 ± 22</td>
<td>3.7 ± 0.3†</td>
<td>0.028 ± 0.009†</td>
<td>0.004 ± 0.0012</td>
</tr>
<tr>
<td>L-NAME</td>
<td>125 ± 22</td>
<td>3.6 ± 0.5†</td>
<td>0.029 ± 0.007†</td>
<td>0.003 ± 0.0008</td>
</tr>
</tbody>
</table>

L-Arginine transport (100 μmol/L, 37°C) in cells preincubated (30 minutes) with 5 mmol/L D-glucose, in absence or presence of 100 μmol/L PD-98059 or 100 μmol/L L-NAME, and exposed (2 minutes) to Krebs containing 5 or 25 mmol/L D-glucose. Values are mean ± SEM, n = 6.

*P < 0.05, †P < 0.05 vs 5 and 25 mmol/L D-glucose, respectively.

NO Involvement
Elevated D-glucose increased eNOS phosphorylation at Ser$^{1177}$ (Figure 3A), l-[3H]citrulline (Figure 3B), and cGMP accumulation (Figure 3C). L-NAME inhibited the effect of D-glucose on cGMP and l-[3H]citrulline formation but did not alter eNOS-Ser$^{1177}$ phosphorylation (not shown). However, wortmannin inhibited D-glucose–induced eNOS phosphorylation at Ser$^{1177}$, cGMP, and l-[3H]citrulline formation. Similar results were found in cells exposed to 25 mmol/L D-glucose for the last 4, 10, or 20 minutes of the 30-minute incubation period with l-[3H]arginine (not shown). Intracellular Ca$^{2+}$ in cells incubated with 25 mmol/L D-glucose for 2 minutes (42 ± 7 nmol/L) was not statistically different ($P > 0.05, n = 125$ cells) from values in cells incubated with 5 mmol/L D-glucose (35 ± 5 nmol/L).

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Figure 2. Effect of D-glucose on kinetic parameters and trans-stimulation of L-arginine transport. A, Saturable l-arginine transport (1 minute, 37°C) in HUVECs incubated (2 minutes) in 5 mmol/L D-glucose (○) or 25 mmol/L D-glucose (●). B, L-arginine transport (100 μmol/L, 1 minute, 37°C) in cells preincubated (2 hours) in medium 199 in the absence (control) or presence of 10 mmol/L L-lysine. Transport assays were performed in cells exposed to 5 mmol/L D-glucose (open bars) or 25 mmol/L D-glucose (filled bars) for the last 2 minutes of the 2-hour incubation period with L-lysine. Values are mean ± SEM (n = 16). *P < 0.04 vs all other values.
TABLE 2. d-Glucose Effect on L-Arginine Transport, [3H]TPP⁺ Influx, and Eₘ in HUVECs

<table>
<thead>
<tr>
<th></th>
<th>L-Arginine Transport, pmol/μg Protein per Minute</th>
<th>[3H]TPP⁺ Influx, pmol/mg Protein per Minute</th>
<th>Eₘ, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mmol/L d-Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>−67.2 ± 0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 ± 0.1*</td>
<td>0.1 ± 0.04*</td>
<td>−8.1 ± 0.5*</td>
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<tr>
<td>Gilbenclamide</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>−63.9 ± 0.6</td>
</tr>
<tr>
<td>Levromakalim</td>
<td>4.6 ± 0.3*</td>
<td>5.6 ± 0.3*</td>
<td>−74.2 ± 0.3*</td>
</tr>
<tr>
<td>Levromakalim + gilbenclamide</td>
<td>1.6 ± 0.2†</td>
<td>1.4 ± 0.6†</td>
<td>−64.5 ± 0.2†</td>
</tr>
<tr>
<td>PD-98059</td>
<td>2.1 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>−64.2 ± 0.5</td>
</tr>
<tr>
<td>L-NAME</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>−65.1 ± 0.3</td>
</tr>
<tr>
<td>SNAP</td>
<td>5.1 ± 0.3*</td>
<td>3.9 ± 0.4*</td>
<td>−76.2 ± 1.0*</td>
</tr>
<tr>
<td>SNAP + PD-98059</td>
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<td>1.9 ± 0.5†</td>
<td>−65.3 ± 1.0†</td>
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<tr>
<td>KT-5823</td>
<td>1.9 ± 0.5</td>
<td>1.9 ± 0.3</td>
<td>−66.8 ± 0.9</td>
</tr>
<tr>
<td>25 mmol/L d-Glucose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.0 ± 0.5*</td>
<td>3.7 ± 0.3*</td>
<td>−77.1 ± 0.2*</td>
</tr>
<tr>
<td>KCl</td>
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<td>0.3 ± 0.03*†</td>
<td>−6.2 ± 0.5†</td>
</tr>
<tr>
<td>Gilbenclamide</td>
<td>2.3 ± 0.4†</td>
<td>2.4 ± 0.4†</td>
<td>−62.7 ± 0.5†</td>
</tr>
<tr>
<td>Levromakalim</td>
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<td>4.7 ± 0.2*</td>
<td>−75.1 ± 0.3*</td>
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<td>Levromakalim + gilbenclamide</td>
<td>2.2 ± 0.3††</td>
<td>1.8 ± 0.3††</td>
<td>−61.9 ± 0.3††</td>
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<td>PD-98059</td>
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<td>1.7 ± 0.1†</td>
<td>−69.9 ± 1.0†</td>
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<tr>
<td>L-NAME</td>
<td>1.4 ± 0.1†</td>
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<td>−65.7 ± 0.7†</td>
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<tr>
<td>SNAP</td>
<td>4.1 ± 0.4*</td>
<td>3.2 ± 0.2†</td>
<td>−78.2 ± 1.0†</td>
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<tr>
<td>SNAP + PD-98059</td>
<td>1.1 ± 0.5††</td>
<td>1.9 ± 0.2††</td>
<td>−65.7 ± 1.0††</td>
</tr>
<tr>
<td>KT-5823</td>
<td>1.9 ± 0.5†</td>
<td>1.9 ± 0.3†</td>
<td>−65.3 ± 0.5†</td>
</tr>
</tbody>
</table>

L-Arginine transport (100 μmol/L), [3H]TPP⁺ influx (46 nmol/L), and Eₘ (whole-cell patch clamp) in cells preincubated (30 minutes) with 5 mmol/L d-glucose and 5.5 mmol/L (control) or 131 mmol/L KCl. Cells in 5.5 mmol/L KCl were preincubated with 10 μmol/L glibenclamide (5 minutes), 1 μmol/L levromakalim (5 minutes), 10 μmol/L PD-98059 (30 minutes), 100 μmol/L L-NAME (30 minutes), 10 μmol/L KT-5823 (30 minutes), or 100 μmol/L SNAP (2 minutes) and exposed (2 minutes) to 5 or 25 mmol/L d-glucose. Values are mean ± SEM, n=17. *P<0.05 vs control in 5 mmol/L d-glucose; †P<0.05 vs corresponding values in SNAP or levromakalim; †‡P<0.05 vs control in 25 mmol/L d-glucose.

2.8 ± 0.4 pmol/μg protein per 30 minutes, respectively), TPP⁺ influx (4.4 ± 0.2 and 4.1 ± 0.3 pmol/mg protein per minute, respectively), or changes in Eₘ (−76 ± 0.3 and −78 ± 0.3 mV, respectively).

Discussion

The present study establishes that d-glucose induces a rapid concentration-dependent stimulation of l-arginine transport in HUVECs. This effect requires NO synthesis associated with increased phosphorylation of eNOS at Ser¹⁷⁷ and activation of p42/p44MAPK and PI3-κ, and it is independent of PKC and intracellular Ca²⁺ changes. These findings provide the first evidence that short-term hyperglycemia activates the l-arginine/NO signaling pathway in human fetal endothelium.

l-Arginine transport is mediated by systems y⁻/CATs, y⁻L₁,₂,₉,₁₀ and b₅ Lester in HUVECs, with the first likely predominating at the physiological concentration of extracellular l-arginine. The cDNAs for four potential human y⁻ transmitters (hCAT-1, hCAT-2B, hCAT-2A, and hCAT-4) have been sequenced.31 l-Arginine transport in HUVECs occurs with relatively high affinity (Kₘ ≈ 80 to 100 μmol/L) and, as confirmed here, is Na⁺ independent and inhibited by membrane depolarization.2,₃,₁₂ Because of their similar kinetic properties, CAT-1 and CAT-2B are hard to distinguish at the functional level.3₁ Our results show that both high-affinity hCAT-1 (Kₘ ≈ 100 to 200 μmol/L) and hCAT-2B (Kₘ ≈ 200 to 400 μmol/L), but not the low-affinity hCAT-2A transporter (Kₘ ≈ 2 to 5 mmol/L), are present in HUVECs, confirming previous reports.3,₁₂,₁₃ CAT-1 is more sensitive than CAT-2B to trans-stimulation by cationic amino acids.3,₁₂,₁₄ When we preloaded HUVECs with l-lysine, l-arginine transport was increased by ≈7-fold in 5 mmol/L d-glucose. However, the l-lysine trans-stimulatory effect was less effective (≈3-fold) in cells exposed for 2 minutes to 25 mmol/L d-glucose. Because 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,2,₁₄ trans-stimulation in HUVECs in 5 mmol/L d-glucose may be preferentially mediated by hCAT-1. The reduced trans-stimulation of transport in high d-glucose may result from a state of maximal activity of l-arginine transporters already induced by l-lysine; therefore, high d-glucose could not further increase l-arginine transport. In addition, the possi-
The ability that L-lysine-stimulated L-arginine transport was due to membrane hyperpolarization is unlikely because $E_m$ was unaltered in L-lysine-preloaded cells.

Long-term incubation (24 hours) of HUVECs with high D-glucose increases $V_{\text{max}}$ for L-arginine transport. The present study shows that acute (2-minute) D-glucose increases $V_{\text{max}}$ for L-arginine transport without altering the apparent $K_m$. As noted above, L-arginine transport is sensitive to changes in extracellular K$^+$ and $E_m$. Because high D-glucose induced membrane hyperpolarization, stimulation of L-arginine transport could result from changes in $E_m$. The stimulatory effect of D-glucose on TPP$^+$ influx and L-arginine transport was blocked by glibenclamide, a K$^+$ ATP channel blocker. In addition, levcromakalim (K$^+$ ATP activator) mimics D-glucose-induced changes in $E_m$, L-arginine transport, and TPP$^+$ influx. K$^+$ ATP channels are expressed in the endothelium and are activated by D-glucose; thus, the effects of D-glucose may involve changes in the activity of glibenclamide-sensitive K$^+$ ATP channels.

D-Glucose (24 hours) also increases eNOS expression and activity in HUVECs. In the present study, eNOS activity was increased in HUVECs exposed for 1 to 5 minutes to high D-glucose, an effect associated with increased phosphorylation of eNOS at Ser$^{1177}$, a residue known to be associated with eNOS activation. The rapid eNOS stimulatory effect of D-glucose was not further increased by longer incubation periods with D-glucose, which could be due to a maximal and sustained activation of eNOS acutely induced by elevated D-glucose. Because D-glucose did not alter basal intracellular...
It is likely that rapid eNOS activation by d-glucose is Ca\(^{2+}\) independent, supporting recent observations of Ca\(^{2+}\)-independent eNOS activation in HUVECs.\(^{37,38}\) p-Glucose-induced phosphorylation of eNOS at Ser\(^{1177}\), L-arginine transport, and TPP\(^{+}\) influx were blocked by wortmannin, suggesting that the PI3-k pathway could be involved in the effects of d-glucose. L-Arginine transport could be determinant for eNOS activity\(^{11}\); however, the possibility that the d-glucose-induced increase of L-citrulline production was due to elevated L-arginine transport seems unlikely, inasmuch as d-glucose-induced NO synthesis was unaltered in the absence of extracellular L-arginine.

L-NAME blocked d-glucose-increased L-arginine transport and NO synthesis in HUVECs. This inhibitor does not alter basal L-arginine transport in the endothelium\(^{2,4,39}\); thus, NO most likely mediates changes in L-arginine transport, as suggested in bovine aortic endothelium.\(^{40}\) This result is similar to that found in HUVECs from patients with gestational diabetes; L-arginine transport in these cells is increased concomitantly with membrane hyperpolarization and NO synthesis, and this increase is inhibited by blocking NO synthase.\(^{2}\) NO causes membrane hyperpolarization in the endothelium,\(^{3,36}\) and NO (from SNAP) has been shown to cause comparable increases in TPP\(^{+}\) influx and L-arginine transport and to cause membrane hyperpolarization to those caused by d-glucose, although SNAP treatment did not further enhance the effects of high d-glucose, in HUVECs. These findings support the hypothesis that NO acutely modulates L-arginine transport by a mechanism that involves membrane hyperpolarization.

NO-altered K\(^{+}\) channel activity may occur by both indirect mechanisms via cGMP and direct NO action on channels.\(^{36}\)
Our results show that the d-glucose increases in t-arginine transport and TPP+ influx were mimicked by dbcGMP and blocked by the PKG inhibitor KT-5823. In addition, d-glucose–induced membrane hyperpolarization was also blocked by KT-5823. Thus, modulation of ion channel activity (and hence, t-arginine transport) could be due to the activation of PKG downstream from NO synthesis.

PKC activity is increased in subjects with diabetes mellitus or in endothelium chronically exposed to high d-glucose.12,16,17 Activation of diacylglycerol/phorbol ester-sensitive PKC isoforms activates eNOS and the NO-dependent increased p42/p44mapk phosphorylation in HUVECs exposed for 24 hours to high d-glucose.3 However, 25 mmol/L d-glucose for 1 to 5 minutes did not alter PKC activity in this cell type, suggesting that the rapid d-glucose effect on t-arginine transport was PKC independent. Because d-glucose induces a rapid (2-minute) p42/p44mapk phosphorylation and because inhibition of the p42/p44mapk phosphorylation by PD-98059 also inhibits the d-glucose increase in TPP+ influx and t-arginine transport, it is likely that p42/p44mapk activation is involved in this pathway. Activation of p42/p44mapk requires PI3-k activity in HUVECs.41 Our results show that d-glucose–induced p42/p44mapk phosphorylation is blocked by wortmannin, suggesting that the d-glucose effect requires PI3-k activity in HUVECs. SNAP-increased p42/p44mapk phosphorylation and t-arginine transport were blocked by PD-98059, complementing results showing that NO, via cGMP, causes rapid p42/p44mapk phosphorylation in the endothelium.4,42 Because d-glucose–induced and NO-induced membrane hyperpolarization are blocked by PD-98059, p42/p44mapk activation could modulate ion channel activity and t-arginine transport in HUVECs.

Elevated d-glucose leads to overproduction of oxygen-derived free radicals in several cell types.11,16,17 We found that SOD activity in HUVECs was unaltered by 25 mmol/L d-glucose and that SOD or α-tocopherol did not block the effects of d-glucose, suggesting that short-term incubation with elevated d-glucose would not generate enough oxygen-derived free radicals to induce changes in the t-arginine/NO pathway in HUVECs.

The present study has established that high d-glucose rapidly activates the t-arginine/NO pathway in HUVECs. The effect of d-glucose involves PI3-k–dependent, but PKC-independent and intracellular Ca2+–independent, eNOS and p42/p44mapk activation. These results complement previous observations in animal models in which elevated plasma d-glucose results in a rapid (seconds to minutes) vasodilatation9,10 and observations of d-glucose–induced vasodilatation in humans.7 Local NO synthesis could be one mechanism by which rapid alterations in plasma d-glucose result in vasodilatation and may have important implications in diabetic patients, in whom plasma d-glucose concentrations may change rapidly.11,12,16,17

Acknowledgments

This study was supported by Fondo Nacional de Ciencia y Tecnología (FONDECYT 1000354 and 7000354), Universidad de Concepción (DIUC 201.084.003-1 (Chile), and The Wellcome Trust (United Kingdom); P. Casanello, J. Parodi, and C. Aguayo hold Beca Docente Universidad de Concepción and CONICYT-PhD (Chile) fellowships. We thank the midwives of the Hospital Regional-Concepción (Chile) labor ward for the supply of umbilical cords and Isabel Jara for secretarial assistance.

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Circ Res. 2003;92:64-72; originally published online November 21, 2002;
doi: 10.1161/01.RES.0000048197.78764.D6

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