Insulin Restores Gestational Diabetes Mellitus—Reduced Adenosine Transport Involving Differential Expression of Insulin Receptor Isoforms in Human Umbilical Vein Endothelium

Francisco Westermeier,1 Carlos Salomón,1 Marcelo González,1,2 Carlos Puebla,1 Enrique Guzmán-Gutiérrez,1 Fredi Cifuentes,1,3 Andrea Leiva,1 Paola Casanello,1 and Luis Sobrevia1

OBJECTIVE—To determine whether insulin reverses gestational diabetes mellitus (GDM)—reduced expression and activity of human equilibrative nucleoside transporters 1 (hENT1) in human umbilical vein endothelium cells (HUVECs).

RESEARCH DESIGN AND METHODS—Primary cultured HUVECs from full-term normal (n = 44) and diet-treated GDM (n = 44) pregnancies were used. Insulin effect was assayed on hENT1 expression (protein, mRNA, SLC29A1 promoter activity) and activity (initial rates of adenosine transport) as well as endothelial nitric oxide (NO) synthase activity (serine1177 phosphorylation, L-citrulline formation). Adenosine concentration in culture medium and umbilical vein blood (high-performance liquid chromatography) as well as insulin receptor A and B expression (quantitative PCR) were determined. Reactivity of umbilical vein rings to adenosine and insulin was assayed by wire myography. Experiments were in the absence or presence of L-N^6-nitro-L-arginine methyl ester (L-NAME; NO synthase inhibitor) or ZM-241385 (an A2A-adenosine receptor antagonist).

RESULTS—Umbilical vein blood adenosine concentration was higher, and the adenosine- and insulin-induced NO/endothelium-dependent umbilical vein relaxation was lower in GDM. Cells from GDM exhibited increased insulin receptor A isoform expression in addition to the reported NO–dependent inhibition of hENT1-adenosine transport and SLC29A1 reporter repression, and increased extracellular concentration of adenosine and NO synthase activity. Insulin reversed all these parameters to values in normal pregnancies, an effect blocked by ZM-241385 and L-NAME.

CONCLUSIONS—GDM and normal pregnancy HUVEC phenotypes are differentially responsive to insulin, a phenomenon where insulin acts as protecting factor for endothelial dysfunction characteristic of this syndrome. Abnormal adenosine plasma levels, and potentially A2A-adenosine receptors and insulin receptor A, will play crucial roles in this phenomenon in GDM.

Diabetes 60:1677–1687, 2011

Gestational diabetes mellitus (GDM) is a syndrome associated with maternal hyperglycemia and defective insulin signaling in the placenta (1–3). GDM leads to fetoplacental vascular endothelial dysfunction (4,5), a condition associated with reduced uptake and subsequent increased extracellular concentration of adenosine, an endogenous purine vasodilator, in primary cultured human umbilical vein endothelial cells (HUVECs) (6,7). Even though adenosine is a well-known vasodilator in the human placental circulation (8) and in other vascular beds (9), no reports have addressed the umbilical plasma level of adenosine in GDM. The adenosine concentration in umbilical vein blood is elevated in pregnancies where the mother is affected by diabetes (10). Because plasma adenosine level is mainly regulated by the capacity of the endothelium to take up and metabolize this nucleoside (3,5,11,12), ectonucleotidases are not expressed in HUVECs (12) and the umbilical sera blood content of adenosine deaminase, an enzyme that metabolizes adenosine, is comparable in GDM and in normal pregnancies (13); a crucial role for nucleoside transporters has been proposed in this cell type in GDM (3,6,14). In HUVECs under physiologic conditions, adenosine uptake is mediated by equilibrative human nucleoside transporters 1 (hENT1), which is inhibited by ≤1 μmol/L nitrobenzylthioinosine (NBTI) and 2 (hENT2), which is inhibited by >1 μmol/L NBTD and cross-inhibited by hypoxanthine (6,7,15). Interestingly, because GDM is associated with nitric oxide (NO)–dependent reduced hENT1-adenosine transport in HUVECs (7), changes in expression and/or activity of hENT1 could result in altered physiologic plasma adenosine concentration, leading to endothelial dysfunction in this syndrome.

Elevated extracellular β-glucose reduces hENT1 expression and activity in a NO–dependent manner in HUVECs, effects reversed by insulin; however, this hormone reduced hENT1 expression and activity in HUVECs cultured at physiologic concentrations of β-glucose (16). Because the deleterious effect of GDM on placental endothelial function most likely results from maternal and fetal hyperglycemia (1,2,17,18) and supraphysiologic umbilical vein blood hyperinsulinemia is detected in GDM (2,19,20), we hypothesize that this hormone will reverse GDM-associated reduced hENT1 expression and activity in HUVECs. Interestingly, because insulin resistance is associated with increased expression of A2A-adenosine receptors in monocyte-derived macrophages.
from subjects with type 2 diabetes (21), we expect adenosine receptors to play a role in the effect of insulin in HUVECs in GDM. Our results show that reduced adenosine transport in HUVECs from GDM pregnancies is reversed by insulin to values in cells from normal pregnancies, where insulin receptor isoforms A (IR-A) and B (IR-B) and A2A-adenosine receptors may play a role.

RESEARCH DESIGN AND METHODS

Study groups. Umbilical cords were collected after delivery from 44 full-term normal or 44 full-term GDM pregnancies. The investigation conformed to the principles outlined in the Declaration of Helsinki. Ethics committee approval was obtained from the Faculty of Medicine of the Pontificia Universidad Católica de Chile, and patients provided informed consent. Patients with basal glycemia <90 mg/dL (i.e., overnight fasting) and >140 mg/dL at 2 h after an oral glucose load (75 g) were diagnosed as having GDM and were treated with diet (Table 1) (22).

Cell culture. Confluent HUVEC primary cultures (passage 3, 37°C, 5% CO2) isolated by collagenase digestion (0.25 mg/mL using Collagenase Type II from Clostridium histolyticum (Boehringer, Mannheim, Germany) were exposed to insulin (0.01–100 μmol/L) in medium 109 (M199; Gibco Life Technologies, Carlsbad, CA) containing 5 mmol/L glucose, 10% newborn calf serum, 10% FCS, 3.2 mmol/L glutamine, and 100 units/mL penicillin-streptomycin (primary culture medium) (16), in the absence or presence of 100 μmol/L N^6-nitro-l-arginine methyl ester (l-NAME; NOS inhibitor) (6.7,16). Cells were cultured in primary culture medium containing 25% newborn calf serum and 25% FCS for 24 h before experiments.

Adenosine transport. Total (overall) 10 μmol/L adenosine transport (i.e., hENT1 + hENT2-mediated) was measured in the absence or presence of S-(4-nitrobenzyl)-6-thio-inosine (NBFI; 1 μmol/L ENTI inhibitor), hypoxanthine (2 mmol/L, ENT2 substrate), or both (7). The difference between total transport and transport in the presence of 1 μmol/L NBFI was defined as ENTI-mediated transport (7.16).

Reverse transcription and quantitative RT-PCR. Total RNA aliquots were reverse-transcribed into cDNA and quantitative RT-PCR in a LightCycler rapid RT-PCR machine (Young, Wokingham, UK) in the presence or absence of 1 μmol/L l-NAME, 100 μmol/L ENT2 inhibitor, or both after 3-s step to 5°C (IR-A), 60°C (IR-B), 56°C (18S), and extension at 72°C (hENT1, 15 s; IR-A, 20 s; IR-B, 25 s; 18S, 10 s) in the presence of 1 μmol/L dithiothreitol. Primer sequences (forward and reverse): hENT1 (sense) 5′-TCTCCAACTCTCGACC-CACCAA-3′, hENT1 (antisense) 5′-CTCGGAGTGTCGTGGACTGACTT-3′, IR-A (sense) 5′-GCTGAGACCTGCTCCCGAGGA-3′, IR-A (antisense) 5′-GAGAGTGGCTGAGCAGCAA-3′, IR-B (sense) 5′-GCTGAGACTCTCCTCGAGGA-3′, IR-B (antisense) 5′-AGATGGCTCTAGGCTCCTCGG-3′, 18S (sense) 5′-TCAAGGAACAGCTGAGGAGG-3′, and 18S (antisense) 5′-GGACATCTAAGGCGATCACA-3′. Expected size products for hENT1 (151 bp), IR-A (210 bp), IR-B (244 bp), and 18S (480 bp) were confirmed in PCR experiments. The 18S RNA number of copies was unaltered (P > 0.05, n = 6) in all experimental conditions (not shown).

Western blotting. Proteins (70 μg) separated by polyacrylamide gel (10%) electrophoresis were probed with primary polyclonal goat anti-hENT1 (1:1000), rabbit anti-eNOS (1:1500), rabbit anti-phosphorylated eNOS at Serine1177 (P ~ Ser1177-eNOS, 1:250) or monoclonal mouse anti-β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, followed by 1-h incubation in Tris buffer saline with Tween0.2% BSA containing secondary horseradish peroxidase-conjugated goat anti-goat, anti-rabbit, or anti-mouse antibodies (Santa Cruz Biotechnology), as described (7,16,23). Proteins were detected by enhanced chemiluminescence at a film exposure of 5 min and quantitated by densitometry.

hENT1 promoter cloning. Genomic DNA was isolated using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI). The sequences ~3196 and ~1670 bp from the ATG translation start codon of the SLC29A1 gene (Genbank AF057360) were PCR-amplified using the Elongase Rapid Amplification System (Invitrogen, Carlsbad, CA) and cloned into the pGL3-basic reporter system (7). The pGL3-hENT1 reporter constructs generated were pGL3-hENT1*-1296 and pGL3-hENT1*-1670.

Transient transfection. Cell suspension (3.2 × 10^6 cells/mL) was mixed with pGL3-hENT1 reporter constructs, pGL3-Basic (empty pGL3 vector), pGL3-Control (simian virus 40 promoter [SV40] pGL3 vector), or the internal transfection control vector pGL2-TK expressing Renilla luciferase (Promega) (7). Cells were electroporated (300 mvolts, 700 μL/mg) and cultured in M199 containing 2% FCS for 48 h before experiments.

Luciferase assay. Firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in a Sirius luminometer (Berthold Detection System, Oak Ridge, TN) (7).

Adenosine measurements by high-performance liquid chromatography. Adenosine concentration in the extracellular medium of cultures of HUVEC and from umbilical vein blood was determined by high-performance liquid chromatography equipped with a fluorescence detector (6.14,24).

Vascular reactivity. Endothelium-dependent relaxation was measured in a myograph for isometric force measurements with optimal internal diameter (OID) adjusted from maximal active response to 62.5 mmol/L KCl (23). Response to insulin

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal pregnancies (n = 44)</th>
<th>GDM pregnancies (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32 ± 2 (26–36)</td>
<td>31 ± 4 (25–33)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156 ± 11 (151–169)</td>
<td>154 ± 9 (149–165)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60 ± 4.1 (50–70)</td>
<td>66 ± 2.4 (52–85)</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>24 ± 1.5 (20–28)</td>
<td>25 ± 0.4 (20–29)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109 ± 7 (105–112)</td>
<td>111 ± 6 (107–114)</td>
</tr>
<tr>
<td>Hemoglobin A1C (%) of total</td>
<td>3.4 ± 0.3 (2.8–4.2)</td>
<td>3.9 ± 0.1* (7.1–13.1)</td>
</tr>
<tr>
<td>Glycemia basal (mg/dL)</td>
<td>86 ± 4 (83–89)</td>
<td>81 ± 6 (78–89)</td>
</tr>
<tr>
<td>Glycemia 2 h after glucose (mg/dL)</td>
<td>105 ± 9 (93–114)</td>
<td>188 ± 12* (155–198)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (range), except for hemoglobin A1C, where values are mean ± SD (range). Glycemia was measured at basal conditions (overnight fasting) and 2 h after an oral load with glucose (75 g) as described in RESEARCH DESIGN AND METHODS. GDM patients were treated with diet. *P < 0.05 vs. values in normal pregnancy.
(0.001–100 nmol/L, 8 h) and adenosine (0.1–1 nmol/L, 3 min) was determined in KCl-preconstricted vessels in the absence or presence of 100 μmol/L L-NAME or 10 nmol/L ZM-241385 (A2A-adenosine receptor antagonist) (6).

**Statistical analysis.** Values are mean ± SEM, with n = 44 different cell cultures (3–4 replicates) from normal or GDM pregnancies. Comparisons between two and more groups were performed by means of the unpaired Student t test and ANOVA, respectively. If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by the multiple-comparison Bonferroni correction test. *P < 0.05 was considered statistically significant.

**RESULTS**

**Patients and newborns.** The study included 44 patients with normal pregnancies and 44 GDM patients treated with diet (Table 1). All pregnancies were singleton, and pregnant women were normotensive, nonsmoking, did not consume alcohol or drugs, and were without intrauterine infection or any other medical or obstetrical complications.

**Adenosine transport.** To check insulin effect on ENT-like activity, we assayed insulin and NBTI (ENTS inhibitor) effect on transport. GDM reduced overall adenosine transport. The effect was reversed by insulin but unaltered by NBTI (Fig. 1A). Insulin increased, but NBTI reduced, overall transport in cells from normal pregnancies. NBTI blocked the insulin effect in both cell types. Insulin reduced hENT1-adenosine transport in normal pregnancies; however, it was reversed in GDM to values in normal pregnancies in the absence of insulin (Fig. 1B). Insulin stimulation of overall transport was concentration-dependent (Fig. 1C), and the insulin required was higher in GDM than in normal pregnancies (Table 2).

To test whether NOS activity was involved, L-NAME (general NOS inhibitor) was used. L-NAME increased overall adenosine transport to comparable values in both cell types in the absence of insulin; however, insulin increased overall adenosine transport only in cells from normal pregnancies (Fig. 1C). Insulin reduced hENT1-adenosine transport in a concentration-dependent manner in normal pregnancies, but it was increased in GDM (Fig. 1D), requiring more insulin in GDM than in normal pregnancies (Table 2). In the absence of insulin, hENT1-adenosine transport was higher, reaching comparable values in the presence of L-NAME, which was unaltered by insulin in both cell types.

**NOS activity and eNOS expression.** To assess the role of eNOS in insulin effect, NOS activity and eNOS expression were measured in response to insulin was assayed. Insulin increased l-citrulline formation in normal pregnancies but reversed GDM-associated increase on l-citrulline formation to values in normal pregnancies (Fig. 2A). Equally, insulin increased L-NAME–inhibitable l-citrulline formation in normal pregnancies to values in GDM in the absence of this hormone. However, the total and L-NAME–inhibitable l-citrulline formation were increased in the absence of insulin in GDM (Fig. 2B).

**FIG. 1.** Effect of insulin on adenosine transport. A: Overall (10 μmol/L) adenosine transport (i.e., hENT1 + hENT2–inhibitable; 87 nmol/1 2,3-[3H]adenosine, 37 Cl/mmol, 2 μCi/mL, 20 s, 22°C) was measured in Krebs (mmol/L: NaCl 131, KCl 5.6, NaHCO3 25, NaH2PO4 1, Hepes 20, CaCl2 2.5, MgCl2 1 [pH 7.4], 37°C) in cells preincubated overnight in primary culture medium containing 2% sera from normal (○) or GDM (●) pregnancies and incubated (8 h) without (–) or with (+) insulin and/or NBTI. *P < 0.05 vs. all other values except in cells from normal pregnancies in the presence of insulin + NBTI and from GDM in presence of insulin. †P < 0.05 vs. all other values in GDM. B: hENT1-mediated adenosine transport derived data in A (see RESEARCH DESIGN AND METHODS) in the absence (control) or presence (8 h) of insulin. Cells were exposed (30 min before transport assays) to Krebs, without or with 1 μmol/L NBTI, 2 μmol/L hypoxanthine, or both, at the cis compartment as [3H]adenosine (6,7,16). *P < 0.05 vs. control in cells from normal pregnancies. †P < 0.04 vs. control in cells from normal and GDM pregnancies. C: Overall adenosine transport as in A in cells from normal (○, □) or GDM (●, ■) pregnancies incubated without (0 nmol/L) or with (8 h) increasing concentrations of insulin in the absence (○, ●) or presence (□, ■) of 100 μmol/L L-NAME. D: hENT1-mediated adenosine transport derived from data in C (see RESEARCH DESIGN AND METHODS). *P < 0.05 vs. corresponding values without insulin. †P < 0.04 vs. corresponding values without insulin in presence of L-NAME. The error bars in the graphs designate the SEM.
formation in GMD was partially (63 ± 5%) reduced by insulin. More insulin was required in GDM than in normal pregnancies (Table 2). Relative eNOS phosphorylation versus total eNOS protein abundance was increased in normal pregnancies, but GDM increase in eNOS phosphorylation was reversed by insulin to values in normal pregnancies in the absence of this hormone (Fig. 2B). The estimated insulin effectiveness (i.e., IC50 GDM/SC50 Normal) in this phenomenon was similar in GDM compared with normal pregnancies (Table 2). Insulin caused a higher increase (2.7 ± 0.2-fold) in phosphorylated compared with total eNOS protein abundance in cells from normal pregnancies (Fig. 2C). However, insulin caused a similar reduction in phosphorylated and total eNOS protein abundance in GDM (Fig. 2D).

Expression of hENT1. Because of the possibility that the insulin effect was due to altered hENT1 expression, protein abundance and mRNA expression of hENT1 was assayed. Insulin reduced hENT1 protein abundance in HUVECs from normal pregnancies to values in GDM, but reversed GDM-associated reduced hENT1 protein abundance to values in normal pregnancies in the absence of this hormone (Fig. 3A). The insulin requirement was higher in GDM than in normal pregnancies (Table 2). l-NAME reversed the insulin effect in normal and GDM pregnancies, reaching hENT1 protein abundance levels higher than in the absence of this hormone (Fig. 3B). In addition, l-NAME increased the effect of insulin in GDM pregnancies. Similar responses were obtained for hENT1 mRNA expression (Fig. 3C and D), and the insulin requirement for this effect was similar in normal and GDM pregnancies (Table 2).

SLC29A1 promoter activity. Having found that insulin and GDM effect are associated with altered hENT1 expression, we then checked whether changes correlated with SLC29A1 expression. Reporter luciferase activity in cells from GDM transfected with pGL3-hENT1−1670, but not for the pGL3-hENT1−1670 construct, was lower compared with normal pregnancies in the absence of insulin (Fig. 4A). Insulin reduced reporter activity in cells from normal pregnancies transfected with GL3-hENT1−3108 but increased GMD-reduced pGL3-hENT1−3108 reporter activity to values in normal pregnancies in the absence of this hormone.

The potential involvement of NOS activity in SLC29A1 expression was then assayed. The insulin effect in cells from normal pregnancies was blocked by l-NAME, reaching values higher than basal reporter activity in cells from normal pregnancies in the absence of this hormone (Fig. 4B). GDM-reduced promoter activity was reversed by l-NAME to values in normal pregnancies, an effect that was higher in cells co-incubated with insulin and l-NAME. In addition, l-NAME increased promoter activity to similar values in both cell types when co-incubated with insulin and l-NAME. However, no significant changes were seen for pGL3-hENT1−1670 reporter activity under these conditions (Fig. 4C).

Extracellular adenosine. Reduced expression of hENT1 results in extracellular adenosine accumulation. Thus, we measured adenosine levels in HUVEC cultures. Extracellular adenosine concentration in cells from GDM pregnancies was higher than in cells from normal pregnancies, an effect blocked by insulin and l-NAME (Fig. 5A). However, insulin increased extracellular adenosine concentration in normal pregnancies, an effect blocked by l-NAME. Parallel assays showed increased adenosine plasma concentration in umbilical vein blood in GDM compared with normal pregnancies.

**Umbilical vein response to adenosine.** Vessel OID was estimated to determine whether the basal tone of human umbilical veins was altered in GDM. GDM was associated with larger umbilical vein rings OID (6.05 ± 0.4 mm) compared with normal pregnancies (4.82 ± 0.3 mm) under.
basal conditions ($P < 0.05, n = 18$). In addition, because the extracellular adenosine level is higher in HUVECs from GDM, it is feasible that adenosine could modulate umbilical vein reactivity. Thus, we assayed adenosine effect and involvement of adenosine receptors on reactivity of human umbilical vein rings. Adenosine caused relaxation of umbilical vein rings, an effect less effective in GDM compared with normal pregnancies (Fig. 5B), which was endothelium-dependent and was partially reduced by ZM-241385 in vessels from normal pregnancies but was abolished in GDM pregnancies (Fig. 5C). The ZM-241385–inhibitable fraction of adenosine effect was similar ($P > 0.05$) in normal and GDM pregnancies ($61 \pm 12$ vs. $71 \pm 14\%$, respectively). Adenosine effect was blocked by L-NAME and caused vasoconstriction higher than the KCl-maximal response in both vessel types (Fig. 5D).

**Umbilical vein response to insulin.** We also assayed insulin effect on reactivity of human umbilical vein rings. Insulin caused concentration-dependent relaxation of umbilical vein rings, an effect less effective in GDM compared with normal pregnancies and abolished by L-NAME (Fig. 6A, Table 2). This inhibitor caused vessel contraction
in all experimental conditions. Insulin effect was also blocked by ZM-241385 in normal or GDM pregnancies, causing similar vasoconstriction in the absence or presence of insulin or L-NAME (Fig. 6 B).

**Expression of insulin receptor isoforms.** Because insulin could signal through two isoforms of insulin receptors in HUVECs, we tested whether IR-A and IR-B expression was altered in GDM. IR-A and IR-B mRNA was detectable in HUVECs, with a higher number of mRNA copies for IR-A compared with IR-B (Fig. 6 C). In addition, IR-A expression, but not IR-B mRNA expression, was higher in cells from GDM compared with normal pregnancies. Insulin reversed the GDM effect on IR-A mRNA expression but did not alter IR-A mRNA expression in normal pregnancies, and neither altered IR-B mRNA expression in both cell types.

**DISCUSSION**

This study shows that GDM-associated reduced adenosine transport in HUVECs is reversed by insulin to values in normal pregnancies, involving re-establishment of hENT1 expression and activity. Insulin also reversed GDM-increased eNOS expression and activity, and GDM-reduced SLC29A1 promoter activity to values in normal pregnancies. However, hENT1 activity and expression as well as SLC29A1 promoter activity were reduced by insulin via NO in HUVEC from normal pregnancies. Extracellular adenosine concentrations in HUVECs and in the umbilical vein blood from GDM were higher than in normal pregnancies. GDM was associated with a higher basal OID in umbilical vein rings compared with normal pregnancies.

Adenosine and insulin caused endothelium-dependent relaxation of umbilical vein rings that was dependent on NOS activity and involved activation of A2A-adenosine receptors. HUVECs express IR-A and IR-B isoforms, of which IR-A mRNA expression was higher in GDM compared with normal pregnancies, an effect also reversed by insulin. Altogether, these results suggest that insulin causes differential responses in HUVECs and umbilical vein reactivity, reversing the GDM-associated phenotype to a normal pregnancy-associated phenotype.

**Adenosine transport.** GDM causes NO-dependent reduced hENT1 expression and activity in HUVEC (7), and insulin increases overall adenosine transport in HUVEC.
We here show that insulin restores hENT1-adenosine transport in HUVECs from GDM pregnancies to values in normal pregnancies, suggesting a potential beneficial action of insulin on human fetal endothelial cell function in GDM. A similar amount of insulin was required to stimulate overall and hENT1-mediated transport (P < 0.05) in cells from normal (SC50 overall/SC50 hENT1 = 1.01 ± 0.02) and GDM pregnancies in the presence of insulin, transfected with the pGL3-hENT123198 construct. B: Reporter construct pGL3-hENT11670 of SLC29A1 promoter assayed as in B in the absence (-) or presence (+) of L-NAME. C: Reporter construct pGL3-hENT11670 of SLC29A1 promoter transfected in cells from normal (□) or GDM (■) pregnancies as in A in the absence (-) or presence (+) of L-NAME. *P < 0.05 vs. all other corresponding values in normal or GDM pregnancies. †P < 0.05 vs. corresponding values in normal or GDM pregnancies in the presence of insulin. Error bars in the graphs designate the SEM.

Interestingly, insulin reduced hENT1-adenosine transport in cells from normal pregnancies, thus suggesting a differential effect of insulin on hENT1 transport activity in HUVECs from normal compared with GDM pregnancies. This finding agrees with the reported insulin effect abolishing GDM-increased L-arginine transport in HUVECs (25), experimental diabetes-increased L-arginine transport in rabbit gastric glands (26) and in rat exocrine pancreas (27), and blocking the high extracellular D-glucose–increased L-arginine transport and eNOS activity in HUVECs from normal pregnancies (23). Thus, the beneficial effect of insulin is not a phenomenon restricted to nucleoside transport or HUVECs. However, nothing more than a cycloheximide-dependent, unveiled post-transductional regulation explaining differential mechanism(s) behind the biologic effects of insulin has been reported for these phenomena (3,25,28). NO involvement. GDM-increased NO synthesis and eNOS expression was reversed by insulin to values in normal pregnancies. However, higher NOS activity and eNOS expression in response to insulin was found in normal pregnancies, confirming previous observations in this cell type (16). Thus, the possibility of a potential differential effect of insulin included the capacity of HUVECs to synthesize NO. Interestingly, NOS activity modulation by insulin was paralleled by similar changes in eNOS phosphorylation.
Insulin-induced relaxation (% of KCl).

**FIG. 5.** Effect of adenosine on human umbilical vein reactivity. **A:** Extracellular concentration of adenosine in primary cultures of HUVECs from normal (○) or GDM (■) pregnancies incubated (8 h) in the absence (–) or presence (+) of insulin and/or L-NAME. Culture medium, mixed with 0.5 mol/L acetate buffer, 1 μmol/L adenosine (internal standard), and 10 μmol/L aqueous (50%) chloroacetaldelye, was incubated (80°C, 1 h), centrifuged (14,000g, 4 min), injected (80 μL) into a fluorescence detector-equipped Isco HPLC system (C18 reverse-phase column, 5-μm particle size, 10 mmol/L citrate-buffer with 4.5% acetonitrile as mobile phase), and run isocratically (1 mL/min) as described (6,16). Error bars in the graphs designate the SEM. **B:** Relaxation of 62.5 mmol/L KCl-preconstricted human umbilical vein rings isolated from normal (○) or GDM (■) pregnancies in response to 1 mmol/L adenosine (1 time 0). A representative record from 19 other different measurements is shown. **C:** Effect of adenosine as in B in vessel rings with intact endothelium (–endothelium) or where the endothelium layer was removed (–endothelium), which were incubated without (□) or with (■) 10 mmol/L ZM-241385. †P < 0.05 vs. corresponding values in the absence of ZM-241385. **D:** Relative response (% of KCl) for GDM (■) or normal (○) pregnancies as in B in the absence (–) or presence (+) of adenosine (3 min) and/or L-NAME (30 min). †P < 0.05 vs. all other corresponding values in normal or GDM pregnancies. Error bars in the graphs designate the SEM.

Interestingly, exposure of primary cultures of HUVECs from GDM to supraphysiological concentrations of D-glucose (25 mmol/L, 24 h) resulted in insulin insensitivity (25). Thus, because a higher mother-to-fetus D-glucose flux occurs in GDM pregnancies, which therefore exposes fetal endothelium to hyperglycemia in the uterus (1,2,17,18), lack of modulation of hENT1 activity by insulin in cells from GDM may result from a long-term exposure of HUVECs to an abnormal, environmental condition. However, this feasibility is unlikely because glycermia in patients was controlled by diet; therefore, maternal hyperglycemia will instead most likely increase in postprandial peaks, perhaps lasting for 2 to 4 h and thus leading to states of

be triggering NO-independent mechanism(s) modulating other than hENT1-adenosine transport. Because insulin causes NO-independent hENT2 activation in HUVECs from normal pregnancies (16), insulin-increased overall adenosine transport due to NO-independent activation of this nucleoside transporter isoform in HUVECs from normal pregnancies is feasible. Insulin did not alter overall, and neither did hENT1-adenosine transport in GDM pregnancies where NOS activity was inhibited; thus, it is suggested that this transporter will be subjected to modulation by insulin in fetal endothelium from pregnancies with this syndrome. At present, additional data on the role of hENT2 are required.

Interestingly, exposure of primary cultures of HUVECs from GDM to supraphysiological concentrations of D-glucose (25 mmol/L, 24 h) resulted in insulin insensitivity (25). Thus, because a higher mother-to-fetus D-glucose flux occurs in GDM pregnancies, which therefore exposes fetal endothelium to hyperglycemia in the uterus (1,2,17,18), lack of modulation of hENT1 activity by insulin in cells from GDM may result from a long-term exposure of HUVECs to an abnormal, environmental condition. However, this feasibility is unlikely because glyceremia in patients was controlled by diet; therefore, maternal hyperglycemia will instead most likely increase in postprandial peaks, perhaps lasting for 2 to 4 h and thus leading to states of
in pregnancies complicated by maternal diabetes (10) and recently suggested to occur in patients with type 2 diabetes (21). Adenosine caused relaxation of umbilical vein rings from GDM, a response less potent compared with vessels from normal pregnancies; therefore, a tonic and close to maximal vasodilatory effect of adenosine due to a supra-physiologic concentration of this nucleoside on this type of vessel in GDM is suggested. This is supported by results showing that the adenosine receptor antagonist ZM-241385 blocked adenosine effect and increased the basal tone in these vessels. Because adenosine caused vasodilatation in human fetoplacental vasculature (8), A2A and A2B adenosine receptors are expressed in HUVECs (30) and are involved in adenosine-induced vasodilatation in the human placenta vasculature (31); a role for these adenosine receptors in the response to adenosine of umbilical veins from normal or GDM pregnancies is likely. This phenomenon could result from reduced adenosine transport because inhibition of hENT1 by NBTI caused vasodilatation in human umbilical vein rings from normal pregnancies (32).

Adenosine-induced vasodilatation most likely required NO because its effect was abolished by l-NAME. Furthermore, l-NAME caused vessel contraction reaching similar values in umbilical vein rings from normal or GDM pregnancies in the absence or presence of adenosine. These findings could result from a maximal inhibition caused by adenosine via increased NO synthesis. In fact, incubation of umbilical vessel rings with ZM-241385 and l-NAME resulted in a similar maximal contraction as seen in response to adenosine and l-NAME, thus supporting this possibility. In addition, because the highly selective A2A-adenosine receptor antagonist ZM-241385 abolished the adenosine effect, these types of receptors may be involved in this response.

Vasodilatation of umbilical vein rings caused by insulin was lower in preparations from GDM compared with normal pregnancies. This finding could result from reduced sensitivity to insulin in GDM (IC50 GDM/IC50 normal = 1.36 ± 0.06), agreeing with a potential placental insulin-resistant state in GDM (2,19,20). Insulin required NO synthesis and most likely involved A2A-adenosine receptor activation to cause vasodilatation because l-NAME and ZM-241385 abolishes its effect. ZM-241385 also led to comparable maximal vessel contraction in vessels from normal and GDM pregnancies; therefore, the insulin effect could be due to an increased adenosine level in these preparations. However, this seems feasible only for vessels from normal pregnancies because the extracellular adenosine concentration was increased by insulin in these vessels but was reduced in preparations from GDM. Thus, the insulin effect in umbilical vein rings is different in GDM than in normal pregnancies, and a mechanism other than NO and/or activation of adenosine receptors for insulin effect is suggested for umbilical veins from GDM. A recent report suggests that A2B-adenosine receptor activation could be a factor reducing insulin sensitivity in mice (21); however, nothing is reported regarding this phenomenon in human endothelium.

Potential insulin receptor isoform involvement. Human placenta expresses at least two subtypes of insulin receptors, IR-A and IR-B (4). We here detected the mRNA for IR-A and IR-B in HUVECs from normal and GDM pregnancies, with IR-A being the predominant isoform in normal compared with GDM pregnancies (IR-A/IR-B number of copies in normal vs. GDM ~1.6). Thus, GDM could be associated with a reduced response to insulin repetitive short-term exposure of HUVECs to elevated d-glucose. In addition, we cannot rule out the possibility that response to insulin of HUVECs from GDM resulted from adaptation to a normal (non-GDM) environment in vitro. However, we speculate on the possibility that chronic short-term exposure to hyperglycemia could be enough to cause a lack or an insufficient response to insulin by the fetal vasculature in the uterus. This is supported by the potential insulin insensitivity suggested in HUVECs from GDM exposed to elevated extracellular d-glucose (25).

Vascular reactivity and adenosine levels. Umbilical vein blood adenosine in GDM is higher than in normal pregnancies, correlating with increased extracellular adenosine concentration in cultures of HUVECs from GDM (6). Thus, reduced adenosine transport by the endothelium could, at least in part, be responsible for the elevated umbilical vein blood adenosine level detected in GDM pregnancies. The latter is also a finding complemented by the elevated umbilical cord plasma adenosine reported

FIG. 6. Effect of insulin on human umbilical vein reactivity. A: Relaxation of 62.5 mmol/L KCl-preconstricted human umbilical vein rings isolated from normal (○, □) or GDM (●, ■) pregnancies incubated (8 h) without (0 mmol/L) or with insulin, in the absence (○, ●) or presence (□, ■) of l-NAME. B: Relaxation of vessel rings with intact endothelium from normal (○, □) or GDM (●, ■) pregnancies as in A, incubated (8 h) in the absence (−) or presence (+) of insulin, ZM-241385, and/or l-NAME. C: Expression of insulin receptor isoforms A (IR-A) and B (IR-B) mRNA relative to 18S rRNA (internal reference) in the number of copies in primary cultures of HUVECs from normal or GDM pregnancies incubated (8 h) in the absence (○) or presence (■) as in A. Error bars in the graphs designate the SEM.

diabetes.diabetesjournals.org DIABETES, VOL. 60, JUNE 2011 1685
is not clear (36). Activation of IR-B by insulin leads to
in patients with insulin resistance, but its role in diabetes
IR-A expression modulation could also modulate hENT1
stored to values in normal cells, suggesting that insulin via
adenosine transport activity. In addition, hENT1 protein
abundance and mRNA expression in GDM cells were re-
stored to levels in normal cells, suggesting that insulin via
IRA expression modulation could also modulate hENT1
expression in HUVECs. Dysregulation of the splicing
of the insulin receptor in target tissues of insulin can occur
in patients with insulin resistance, but its role in diabetes
is not clear (36). Activation of IR-B by insulin leads to
preferential activation of a metabolic rather than a mito-
egenic signaling pathway in response to activation of IR-B
in the R’ mouse embryonic fibroblast cell line (37). Thus,
a potentially differential cell-signaling pathway triggered
by activation of insulin receptor subtypes in HUVECs
from GDM (preferentially IR-A) and normal pregnancies
is feasible.

In summary, GDM is associated with abnormal handling
of adenosine by the human umbilical vein endothelium,
serious that could result in an elevated concentra-
tion of this nucleoside in the umbilical vein blood.
Umbilical vein rings exhibited a larger internal diameter
in GDM than in normal pregnancies, suggesting that in
this syndrome, adenosine may induce a tonic umbilical
vein dilatation. We speculate on the possibility that the
elevated adenosine level in the fetal blood in GDM
could be due to reduced hENT1 transport activity by the endo-
thelium. Thus, it is feasible that a correction of the altered
activity (and perhaps expression) of this type of nucleo-
side transporter could help to reduce the chance of the
actual outcome of macrosomic newborns in GDM by lim-
iting the resulting oversupply of metabolic substrates to the
fetus.

Adenosine and insulin caused A2A-adenosine receptor-
mediated relaxation of human umbilical vein rings that
was lower in GDM compared with normal pregnancies.
The GDM-associated increase in extracellular adenosine
concentration and the reduction of hENT1-mediated adeno-
sine transport and expression (including SLC29A1 tranz-
scriptional activity) in HUVECs were reversed by insulin
via a mechanism that included the re-establishment of
this syndrome-induced eNOS activity and expression to
levels in cells from normal pregnancies. Thus, the GDM
phenotype in HUVECs could be reversed to a normal phe-
notype by insulin through modulation of extracellular
adenosine levels and its A2A-adenosine receptor–mediated
biologic effects. In addition, because HUVECs from GDM
preferentially express the A isoform of insulin receptors
(isoform associated with insulin resistance) (33–35) com-
pared with cells from normal pregnancies, a crucial role
for this type of insulin receptor in the fetoplacental vas-
cular endothelial cells from GDM in this phenomenon is
suggested.

ACKNOWLEDGMENTS
This study was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1110977, 1070865,
1080534, 11100192), Chile; the Programa de Investigación Interdisciplinario (PIA) from the Comisión Nacional de
Investigación en Ciencia y Tecnología (CONICYT; Anillos ACT-73); Fellowship Apoyo de Tesis CONICYT (AT-24090190,
AT-24100210), Chile; Dirección de Investigación (DI-1339-07); Vicerrectoría Académica (Anillos ACT-73) postdoctoral re-
search associate at CMPL-PRL; Pontificia Universidad Católica de Chile, Universidad de Antofagasta, Antofagasta,
Chile; and Dirección de Investigación (DIU 210.033.103-1.0), Universidad de Concepción, Concepción, Chile. F.W.,
C.S., E.G.-G., and C.P. hold CONICYT-PhD (Chile) fellowships. C.S. held a Faculty of Medicine, Pontificia Universidad
Católica de Chile-PhD fellowship.

No potential conflicts of interest relevant to this article were
reported.

F.W. contributed to generating experimental data and
obtaining patients’ clinical data, wrote the manuscript, and
drew the figures. C.S. contributed to generating experi-
mental data and obtaining patients’ clinical data. M.G. and
C.P. contributed to discussion and reviewed and edited the
manuscript. E.G.-G. contributed to discussion and obtain-
ing patients’ clinical data; wrote, reviewed, and edited the
manuscript; and drew the figures. F.C. contributed to gen-
erating experimental data. A.L. contributed to generating
experimental data and obtaining patients’ clinical data,
wrote the manuscript, and drew the figures. P.C. contrib-
uted to discussion and reviewed and edited the manuscript.
L.S. contributed to discussion; wrote, reviewed, and edited
the manuscript; and drew the figures.

The authors thank Ninoska Muñoz from the Cellular
and Molecular Physiology Laboratory at the Division of Obstet-
rics and Gynecology, Faculty of Medicine, Pontificia Uni-
versidad Católica de Chile, for excellent secretarial assistance,
and the personnel of the Hospital Clínico Pontificia Univer-
sidad Católica de Chile labor ward for supply of placenta.

REFERENCES
1. Metzger BE, Buchanan TA, Coustan DR, et al. Summary and recom-
endations of the Fifth International Workshop-Conference on
Gestational Diabetes Mellitus. Diabetes Care 2007;30(Suppl. 2):S251–
S260
2. Colomiere M, Permezel M, Riley C, Desoye G, Lappas M. Defective insulin
signaling in placenta from pregnancies complicated by gestational diabetes
rovascular and microvascular endothelial dysfunction in gestational di-
betes. Placenta 2011;32(Suppl. 2):S159–S164
action on the human placental endothelium in normal and diabetic preg-
porters in fetal endothelial dysfunction in diabetes mellitus and hyper-
gestational diabetes-induced L-arginine transport and nitric oxide synthe-
promoter activity and adenosine transport involving transcription factor
complex hICCHP-C/EBPalpha in human umbilical vein endothelial cells
from gestational diabetes. Cardiovasc Res 2010;86:45–54
8. Read MA, Boura AL, Walters WA. Vascular actions of purines in the foetal
9. Eltzschig HK. Adenosine: an old drug newly discovered. Anesthesiology
2009;111:904–915
10. Maguire MH, Szabó I, Valkó IE, Finley BE, Bennett TL. Simultaneous
measurement of adenosine and hypoxanthine in human umbilical cord

1686 DIABETES, VOL. 60, JUNE 2011 diabetes.diabetesjournals.org


34. Savkur RS, Philips AV, Cooper TA. Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nat Genet 2001;28:40–47

