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

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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 (serine^{1177} 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^G-nitro-L-arginine methyl ester (L-NAME; NO synthase inhibitor) or ZM-241385 (an A_{2A}-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 A_{2A}-adenosine receptors and insulin receptor A, will play crucial roles in this phenomenon in GDM. ***Diabetes* 60:1677–1687, 2011**

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Received 9 February 2011 and accepted 21 March 2011.

DOI: 10.2337/db11-0155

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 vasoactive nucleoside, 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), ectonucleosidases 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 NBTI 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 D-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 D-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 A_{2B}-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 A_{2A}-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% CO_2) isolated by collagenase digestion (0.25 mg/mL) using Collagenase Type II from *Clostridium histolyticum* (Boehringer, Mannheim, Germany) were exposed to insulin (0.001–10 nmol/L, 8 h) in medium 199 (M199; Gibco Life Technologies, Carlsbad, CA) containing 5 mmol/L D-glucose, 10% newborn calf serum, 10% FCS, 3.2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin (primary culture medium) (16), in the absence or presence of 100 μmol/L L-N^G-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor) (6,7,16). Cells were cultured in primary culture medium containing 0.25% newborn calf serum and 0.25% 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 (NBTI; 1 μmol/L, ENT1 inhibitor), hypoxanthine (2 mmol/L, ENT2 substrate), or both (7). The difference between total transport and transport in the presence of 1 μmol/L NBTI was defined as ENT1-mediated transport (7,16).

Reverse transcription and quantitative RT-PCR. Total RNA aliquots were reversed-transcribed into cDNA and quantitative RT-PCR in a LightCycler rapid thermal cycler (Roche Diagnostics, Lewes, U.K.) was as described (7,16,23). HotStart *Taq* DNA polymerase was activated (15 min, 95°C), and assays included a 95°C denaturation for 15 s, annealing for 20 s at 58°C (hENT1), 60°C (IR-A), 60°C (IR-B), 56°C (18S), and extension at 72°C (hENT1, 15 s; IR-A, 20 s; IR-B, 20 s; and 18S, 10 s). Fluorescent product was detected after 3-s step to 5°C below the product melting temperature (T_m). Product specificity was confirmed by agarose gel electrophoresis (2% w/v) and melting curve analysis. The product T_m values were 79.5°C for hENT1, 87.2°C for IR-A, 87.6°C for IR-B, and 82.4°C for 18S. hENT1, IR-A, IR-B, and 18S standards were prepared as described (16,23).

Oligonucleotide primers were hENT1 (sense) 5′-TCTCCAACTCTCAGCCCACCAA-3′, hENT1 (antisense) 5′-CCTGCGATGCTGGACTTGACCT-3′, IR-A (sense) 5′-GCTGAAGCTGCCCTCGAGGA-3′, IR-A (antisense) 5′-CGAGATGGCCTGGGGACGAA-3′, IR-B (sense) 5′-GCTGAAGCTGCCCTCGAGGA-3′, IR-B (antisense) 5′-AGATGGCCTAGGGTCCTCGG-3′, 18S (sense) 5′-TCAAGAACGAAAGCTGGAGG-3′, and 18S (antisense) 5′-GGACATCTAAGGGCATCACA-3′. Expected size products for hENT1 (151 bp), IR-A (210 bp), IR-B (244 bp), and 18S (489 bp) were confirmed in PCR experiments. The 18S rRNA 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 Serine^{1177} (P ~ Ser^{1177}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 Tween/0.2% 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 −3198 and −1670 bp from the ATG translation start codon of the *SLC29A1* gene (GenBank: AF495730) were PCR-amplified using the Elongase Enzyme System (Invitrogen, Carlsbad, CA) and cloned into the pGL3-basic reporter system (7). The pGL3-hENT1 reporter constructs generated were pGL3-hENT1^{-3198} 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 pRL-TK expressing *Renilla* luciferase (Promega) (7). Cells were electroporated (300 mvolts, 700 μF, 5–10 ms; Gene Pulser II System, BioRad, Hercules, CA) 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 (6,16,24).

L-[^{3}H]Citrulline assay. L-[^{3}H]Citrulline formation from L-[^{3}H]arginine (4 μCi/mL, 30 min, 37°C) in the absence or presence of 100 μmol/L L-NAME was used to estimate NOS activity (23).

Vascular reactivity. Ring segments (2–4 mm length) from human umbilical veins with and without endothelium were mounted 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 1
Clinical characteristics of patients and newborns

Variables	Normal pregnancies (n = 44)	GDM pregnancies (n = 44)
Maternal variables		
Age (years)	32 ± 2 (26–36)	31 ± 4 (25–33)
Height (cm)	156 ± 11 (151–169)	154 ± 9 (149–165)
Weight (kg)	60 ± 4.1 (50–70)	66 ± 2.4 (52–85)
BMI (kg/m^2)	24 ± 1.5 (20–28)	25 ± 0.4 (20–29)
Systolic blood pressure (mmHg)	109 ± 7 (105–112)	111 ± 6 (107–114)
Hemoglobin A_{1c} (% of total)	3.4 ± 0.3 (2.8–4.2)	7.9 ± 0.1* (7.1–13.1)
Glycemia basal (mg/dL)	86 ± 4 (83–89)	81 ± 6 (78–89)
Glycemia 2 h after glucose (mg/dL)	105 ± 9 (93–114)	188 ± 12* (155–198)
Newborn variables		
Sex (male/female)	27/17	23/21
Gestational age (weeks)	38.2 ± 0.3 (37.6–38.5)	38.3 ± 0.2 (38.0–38.6)
Birth weight (g)	3,387 ± 55 (3,102–3,441)	4,644 ± 63* (4,451–5,309)
Height (cm)	48.7 ± 4.1 (44–53)	51.2 ± 3.6* (48–52)
Ponderal index (g/cm^3 × 100)	3.03 ± 0.2 (2.97–3.09)	3.90 ± 0.2* (3.55–4.34)
Umbilical vein D-glucose (mmol/L)	2.9 ± 0.3 (2.6–3.3)	3.2 ± 0.4 (2.55–4.34)

Data are presented as mean ± SEM (range), except for hemoglobin A_{1c}, 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 mmol/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 (A_{2A}-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 ENTs-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. 1*A*). 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. 1*B*). Insulin stimulation of overall transport was concentration-dependent (Fig. 1*C*), 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. 1*C*). Insulin reduced hENT1-adenosine transport in a concentration-dependent manner in normal pregnancies, but it was increased in GDM (Fig. 1*D*), 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 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. 2*A*). 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

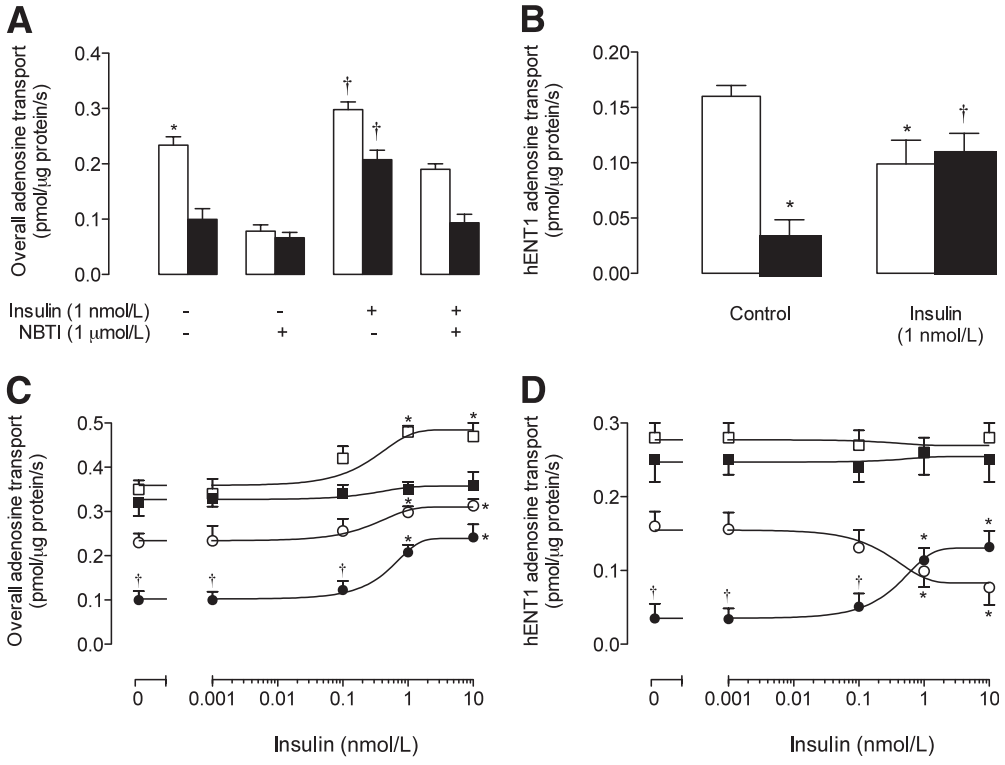

FIG. 1. Effect of insulin on adenosine transport. *A*: Overall (10 μmol/L) adenosine transport (i.e., hENT1 + hENT2–mediated; 87 nmol/l 2,3-[^{3}H] adenosine, 37 Ci/mmol, 2 μCi/mL, 20 s, 22°C) was measured in Krebs (mmol/L: NaCl 131, KCl 5.6, $NaHCO_3$ 25, NaH_2PO_4 1, Hepes 20, $CaCl_2$ 2.5, $MgCl_2$ 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 from 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 mmol/L hypoxanthine, or both, at the *cis* compartment as [^{3}H]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.

TABLE 2
Half-maximal effects of insulin on biologic parameters

Variable	Normal		GDM	
	SC_{50}	IC_{50}	SC_{50}	IC_{50}
Overall adenosine transport				
Control	0.25 ± 0.07	—	0.53 ± 0.06*	—
L-NAME	0.29 ± 0.07	—	NR	—
hENT1 adenosine transport				
Control	—	0.26 ± 0.06	0.45 ± 0.06*	—
L-NAME	NR	NR	NR	NR
hENT1 expression				
Protein abundance	—	0.33 ± 0.04	0.67 ± 0.08*	—
mRNA copies	—	0.31 ± 0.05	0.29 ± 0.05	—
eNOS expression and activity				
L-Citrulline formation	0.22 ± 0.04	—	—	0.43 ± 0.05*
P ~ Ser^{1177}-eNOS/total eNOS	0.35 ± 0.07	—	—	0.32 ± 0.05
P ~ Ser^{1177}-eNOS	0.37 ± 0.05	—	—	0.29 ± 0.04
Total eNOS	0.39 ± 0.04	—	—	0.31 ± 0.05
	EC_{50}		EC_{50}	
Umbilical vein ring dilatation	0.75 ± 0.02		1.02 ± 0.03*	

Data are presented as mean ± SEM. NR, no response. Values for half-maximal stimulatory (SC_{50}) or inhibitory (IC_{50}) effect, or effective concentration (EC_{50}) of insulin are given in nmol/L in HUVECs or umbilical vein rings from normal or GDM pregnancies. Primary cultures of cells were exposed (8 h) to varying concentrations of insulin (0.001–10 nmol/L) in culture medium without (control) or with 100 μmol/L L-NAME. Cells in passage 2 were used for assays of overall and hENT1-mediated adenosine transport (10 μmol/L adenosine, 2 μCi/ml [^{3}H]adenosine, 20 s, 37°C), hENT1 protein abundance, mRNA number of copies, total (total eNOS) and serine^{1177} phosphorylated (P ~ Ser^{1177}-eNOS) eNOS, and L-NAME–inhibited fraction of L-[^{3}H]citrulline formation from L-[^{3}H]arginine (4 μCi/mL L-[^{3}H]arginine, 100 μmol/L L-arginine, 30 min, 37°C). Insulin effect was also assayed in human umbilical vein rings. *$P < 0.05$ vs. insulin effect (SC_{50}, IC_{50}, or EC_{50}) in normal pregnancy.

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. 2*B*). The estimated insulin effectiveness (i.e., IC_{50} GDM/SC_{50} 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. 2*C*). However, insulin caused a similar reduction in phosphorylated and total eNOS protein abundance in GDM (Fig. 2*D*).

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. 3*A*). 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. 3*B*). In addition, L-NAME increased the effect of insulin in GDM pregnancies. Similar responses were obtained for hENT1 mRNA expression (Fig. 3*C* 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^{-3198}, but not for the pGL3-hENT1^{-1670} construct, was lower compared with normal pregnancies in the absence of insulin (Fig. 4*A*). Insulin reduced reporter activity in cells from normal pregnancies transfected with GL3-hENT1^{-3198} but increased GDM-reduced pGL3-hENT1^{-3198} 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. 4*B*). 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. 4*C*).

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. 5*A*). 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

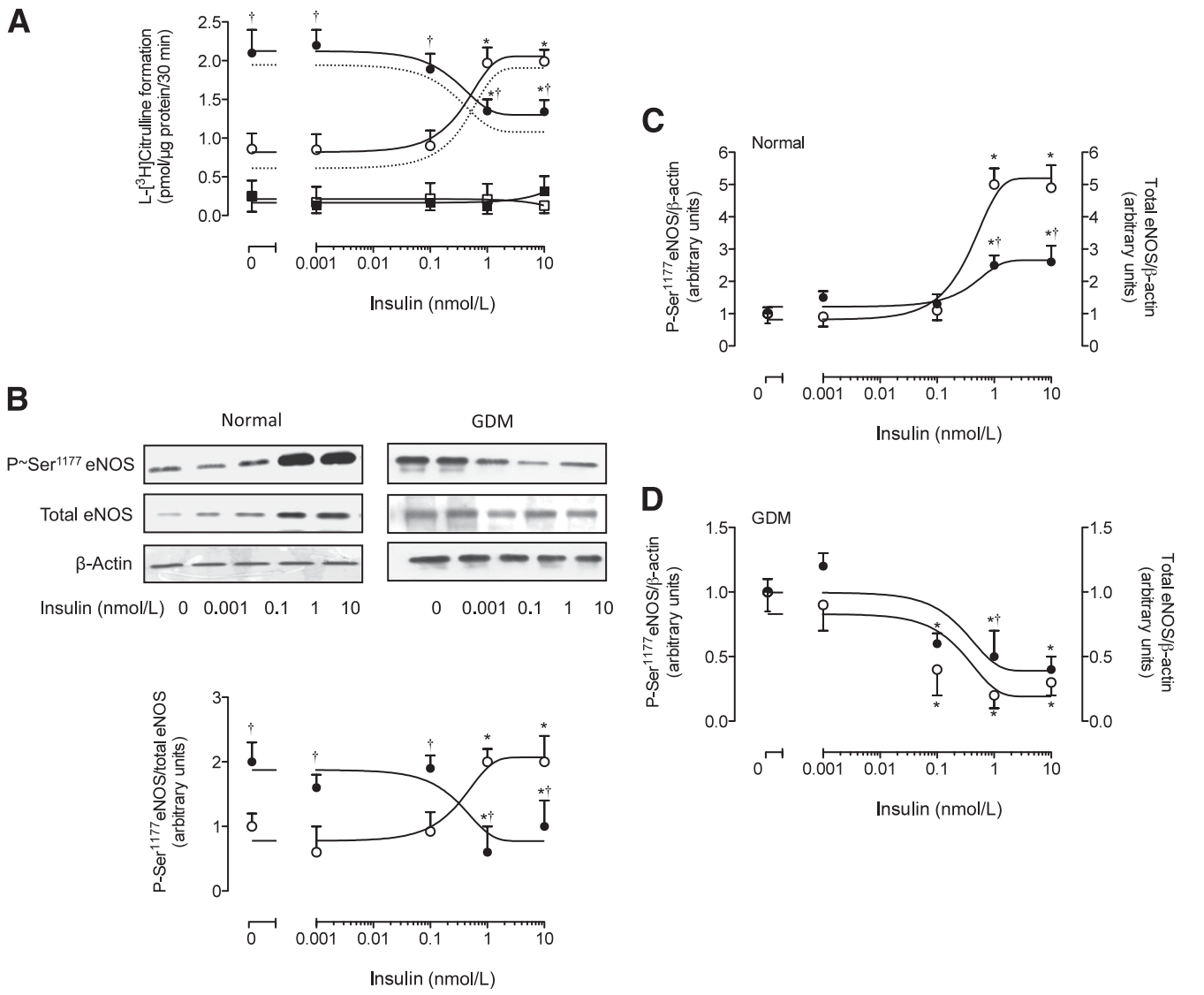

FIG. 2. Effect of insulin on NOS activity. *A*: L-[^{3}H]Citrulline formation from L-[^{3}H]arginine (100 µmol/l L-arginine, 4 µCi/ml L-[^{3}H]arginine, 30 min, 37°C) measured in HUVECs from normal (○, □) or GDM (●, ■) pregnancies incubated (8 h) without (0 nmol/L) or with insulin in the absence (○, ●) or presence (□, ■) of 100 µmol/L L-NAME. After the incubation period, cells were digested (95% formic acid) and passed through a cation ion-exchange resin Dowex-50 W (50 × 8–200), and L-[^{3}H]citrulline was determined in H_2O eluate as described (16,22). Dotted lines represent the L-NAME–inhibited fraction of L-[^{3}H]citrulline formation. All values in the presence of L-NAME were significantly different ($P < 0.03$) from corresponding values in the absence of this inhibitor in cells from normal or GDM pregnancies. *B*: Western blot for total eNOS and Ser^{1177}-phosphorylated eNOS (P ~ Ser^{1177}eNOS) protein abundance in cells from normal or GDM pregnancies incubated (8 h) without (0 nmol/L) or with insulin. Proteins (70 µg) were separated by PAGE (10%) and transferred to Immobilon-P polyvinylidene difluoride membranes. Proteins were detected by enhanced chemiluminescence (5-min film exposure time) and quantitated by densitometry, as described (7,16,22). β-Actin was the internal control. *Lower panel*: P ~ Ser^{1177}eNOS/total eNOS ratio densitometries derived from data in cells from normal (○) or GDM (●) pregnancies, normalized to 1 in cells from normal pregnancies in the absence of insulin. *C*: P ~ Ser^{1177}eNOS/β-actin (○) and total eNOS/β-actin (●) ratio densitometries derived from data in cells from normal pregnancies, normalized to 1 in the absence of insulin. *D*: P ~ Ser^{1177}eNOS/β-actin and total eNOS/β-actin ratio densitometries derived from data in cells from GDM pregnancies, normalized to 1 in the absence of insulin. *$P < 0.05$ vs. corresponding values in the absence of insulin. †$P < 0.05$ vs. corresponding values in cells from normal pregnancies. The error bars in the graphs designate the SEM.

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. 5*B*), which was endothelium-dependent and was partially reduced by ZM-241385 in vessels from normal pregnancies but was abolished in GDM pregnancies (Fig. 5*C*). The ZM-241385–inhibitable fraction of adenosine effect was similar ($P > 0.05$) in normal and GDM pregnancies (61 ± 12 vs. 71 ± 14%, respectively). Adenosine effect was blocked by L-NAME and caused vasoconstriction higher than the KCl-maximal response in both vessel types (Fig. 5*D*).

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. 6*A*, Table 2). This inhibitor caused vessel contraction

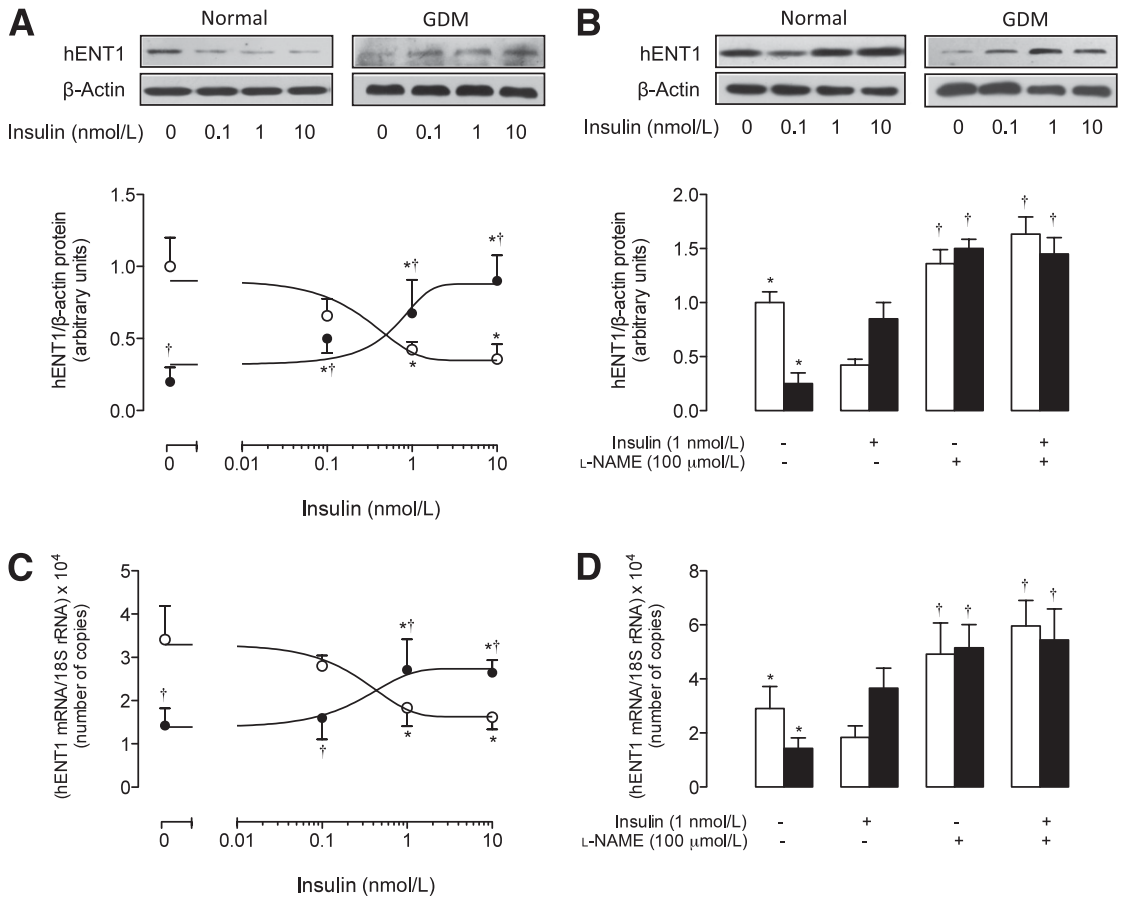

FIG. 3. Effect of insulin on hENT1 expression. ***A*****: Western blot for hENT1 protein abundance in cells from normal or GDM pregnancies incubated (8 h) without (0 nmol/L) or with insulin. β-Actin was the internal control.** ***Lower panel*****: hENT1/β-actin ratio densitometries from data in cells from normal (○) or GDM (●) pregnancies, normalized to 1 in cells from normal pregnancies in the absence of insulin.** ***B*****: Western blot for hENT1 protein abundance in cells incubated (8 h) in the absence (–) or presence (+) of insulin and/or L-NAME. β-Actin was the internal control.** ***Lower panel*****: hENT1/β-actin ratio densitometries were derived from data in cells from normal (□) or GDM (■) pregnancies, normalized to 1 in cells from normal pregnancies in the absence of insulin.** ***C*****: Expression of hENT1 mRNA relative to 18S rRNA (internal reference) in number of copies in cells as in** ***A*****. Quantitative RT-PCR experiments were done in a reaction mix containing 0.5 μmol/L primers, and deoxyribonucleotides triphosphate,** ***Taq*** **DNA polymerase, and reaction buffer provided in the QuantiTect SYBR Green PCR Master Mix (Quiagen, Crawley, U.K.; see RESEARCH DESIGN AND METHODS).** ***D*****: Expression of hENT1 mRNA in number of copies in cells as in** ***B*****. In** ***A*** **and** ***C*****, *P < 0.05 vs. corresponding values in the absence of insulin, †P < 0.05 vs. corresponding values in cells from normal pregnancies. In** ***B*** **and** ***D*****, *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.**

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 A_{2A}-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

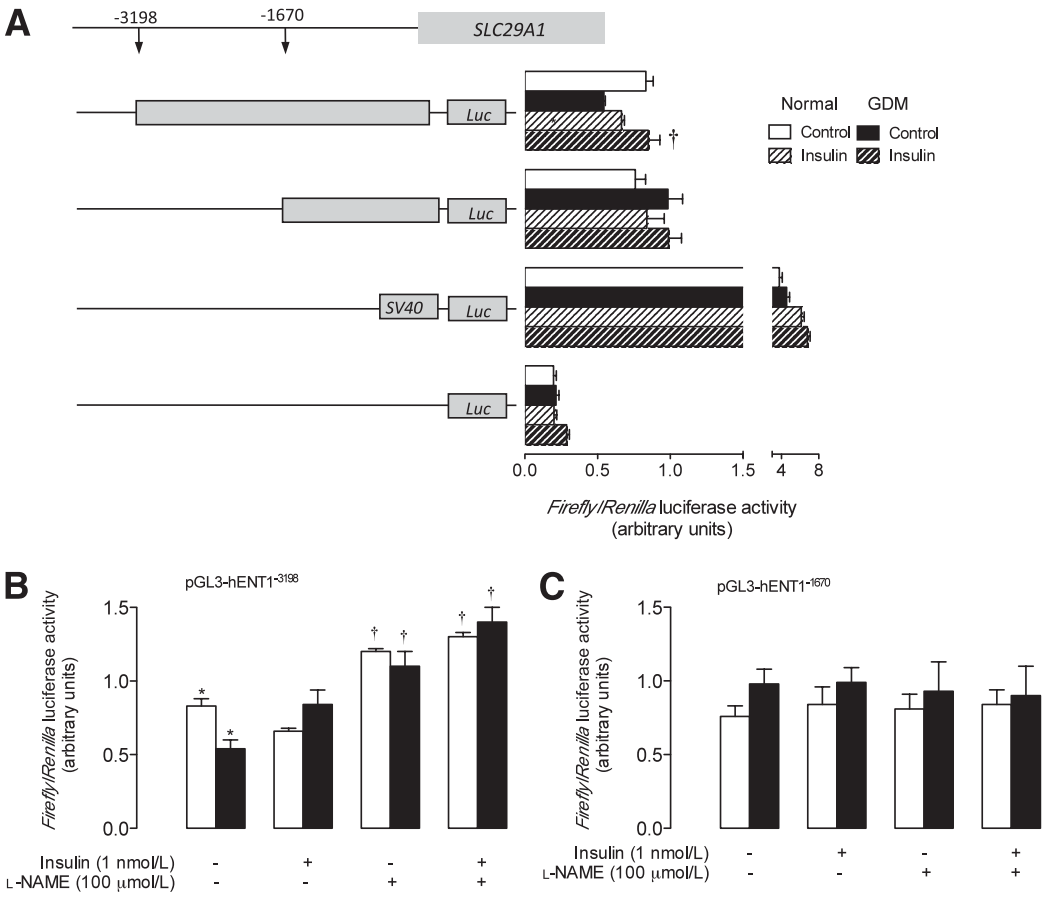

FIG. 4. Effect of insulin on *SLC29A1* (hENT1) promoter activity. *A*: Luciferase (*Luc*) reporter constructs containing two truncations of *SLC29A1* promoter (−3198 and −1670 bp from ATG) were transfected in HUVECs from normal (□) or GDM (■) pregnancies, along with *Renilla* reporter plasmid, and assayed for *Firefly* and *Renilla* luciferase activity, respectively. Results depict the ratio of *Firefly/Renilla* luciferase activity in cells from normal or GDM pregnancies. After 36 h of transfection, cells were incubated for a further 12 h in the absence of insulin (control) or for the last 8 h of the 12-h incubation period in the presence of 1 nmol/L insulin (white [normal] and black [GDM] dashed bars, respectively). Cells were also transfected with the empty pGL3-basic vector or pGL3-control vector (SV40 pGL3) as negative or positive controls, respectively (see RESEARCH DESIGN AND METHODS). *$P < 0.05$ vs. normal pregnancies in the absence of insulin. †$P < 0.05$ vs. normal pregnancies in the absence of insulin or GDM pregnancies in the presence of insulin, transfected with the pGL3-hENT1^{-3198} construct. *B*: Reporter construct pGL3-hENT1^{-3198} of *SLC29A1* promoter transfected in cells from normal (□) or GDM (■) pregnancies as in *A* in the absence (–) or presence (+) of L-NAME. *C*: Reporter construct pGL3-hENT1^{-1670} of *SLC29A1* promoter assayed as in *B*. In *B* and *C*, *$P < 0.05$ 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.

from normal pregnancies (16). 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 (SC_{50} overall/SC_{50} hENT1 = 1.01 ± 0.02) and GDM pregnancies (SC_{50} overall/SC_{50} hENT1 = 1.05 ± 0.03), which suggests insulin action could be due to preferential (if not exclusive) modulation of hENT1 rather than other nucleoside membrane transport mechanism(s). However, a potential increased insulin-resistant state of HUVECs from GDM, as reported for the human placenta in GDM (2), is feasible because insulin was more effective in stimulating overall adenosine transport in cells from normal compared with GDM pregnancies (SC_{50} GDM/SC_{50} normal ~2.12). Because fetal insulin plasma level is increased in GDM (2,19,20), the latter could result as a compensatory mechanism to a defective insulin-induced fetoplacental endothelium signaling.

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

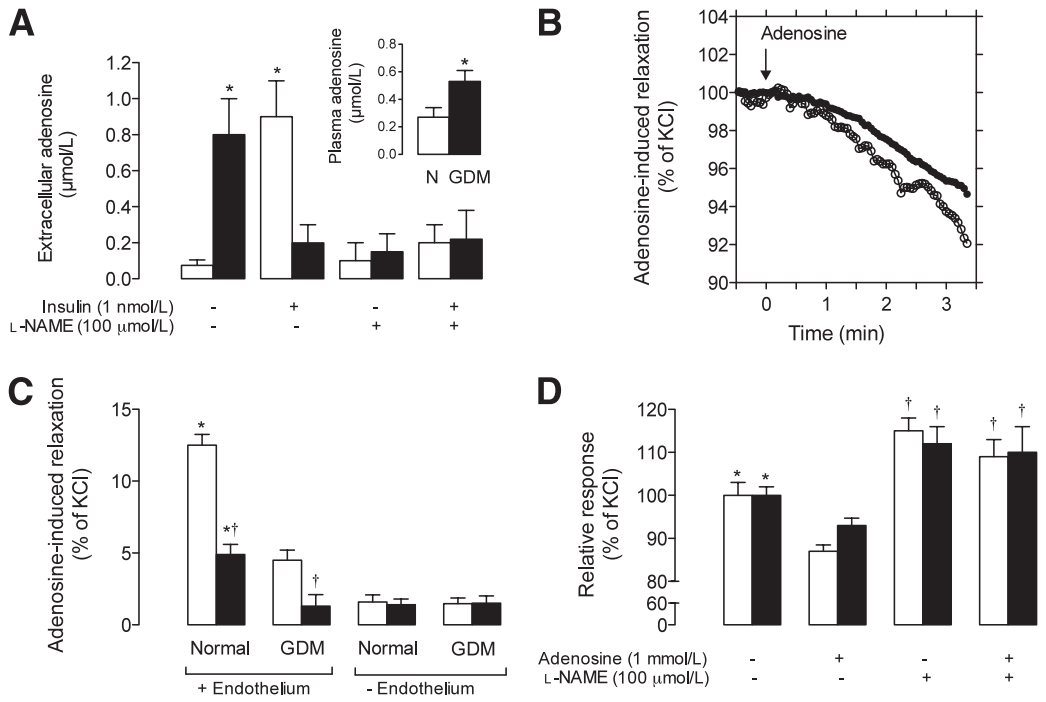

FIG. 5. Effect of adenosine on human umbilical vein reactivity. *A*: Extracellular concentration of adenosine in primary cultures of HUVECs from normal (□) or GDM (■) pregnancies incubated (8 h) in the absence (–) or presence (+) of insulin and/or L-NAME. Culture medium, mixed with 0.5 mol/L acetate buffer, 1 μmol/L adenosine (internal standard), and 10 μmol/L aqueous (50%) chloroacetaldehyde, was incubated (80°C, 1 h), centrifuged (14,000*g*, 4 min), injected (80 μL) into a fluorescence detector-equipped Isco HPLC system (C_{18} 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). *Insert* shows adenosine concentration in human umbilical vein blood for normal (N) or GDM pregnancies. For umbilical vein blood adenosine, blood samples, mixed with 10 μmol/L erythro-9-(2-hydroxy-3-nonyl) adenine, 10 μmol/L NBTI, 1 mmol/L dilazep, 2 μg/mL indomethacin, and 40 μmol/L *O,O'*-bis(2-aminoethyl) ethyleneglycol-*N,N,N'-N'*-tetraacetic acid, taken at birth were centrifuged (14,000*g*, 1 min), deproteinated (50% trichloroacetic acid), and centrifuged (5 min) to obtain a supernatant that was neutralized (100 μL 3.3 N potassium hydroxide). Adenine nucleotides were extracted (1 mol/L zinc sulfate, saturated barium hydroxide, centrifugation at 14,000*g*, 5 min), and adenosine was converted to ethenoadenosine (440 mmol/L chloroacetaldehyde, 80°C, 1 h) for HPLC analysis (23). *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 (↓ 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 nmol/L ZM-241385. †*P* < 0.05 vs. corresponding values in the absence of ZM-241385. *D*: Relaxation of vessel rings with intact endothelium from normal (□) or GDM (■) pregnancies as in *B* in the absence (–) or presence (+) of adenosine (3 min) and/or L-NAME (30 min). †*P* < 0.05 vs. corresponding values in the presence of adenosine. *All panels*: **P* < 0.05 vs. all other corresponding values in normal or GDM pregnancies. Error bars in the graphs designate the SEM.

at Ser^{1177} (i.e., indicative of eNOS activation) (29) in cells from GDM and normal pregnancies, suggesting that modulation of eNOS activity rather than expression is crucial for insulin effect in HUVECs from normal and GDM pregnancies. Insulin-caused eNOS post-transductional modifications result in opposite responses in these two types of cells. However, nothing is yet reported regarding a mechanism for this phenomenon in fetal endothelium (3,28).

We recently showed that reduced *SLC29A1* gene expression leads to lower hENT1-adenosine transport in HUVECs from GDM via a NO-dependent mechanism, involving activation of the hC/element–binding protein (EPB) homologous protein 10 (CHOP)–C/EBP-α transcription factors complex (7). Because we found that insulin *1*) reversed the GDM-reduced *SLC29A1* promoter activity of the construct containing hCHOP-C/EBP-α consensus sequence, *2*) reversed GDM-increased NO synthesis, and *3*) −1845G>T and −1844C>A mutations in *SLC29A1* blocks the NO/hCHOP-C/EBPα–dependent GDM repressive effect on expression (7), insulin action likely involves this complex of transcription factors in its potential beneficial effects in GDM.

Interestingly, insulin increased overall but not hENT1-adenosine transport in the presence of a NOS inhibitor in cells from normal pregnancies. Thus, insulin would also 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.

Interestingly, exposure of primary cultures of HUVECs from GDM to supraphysiologic 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 glycemia 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

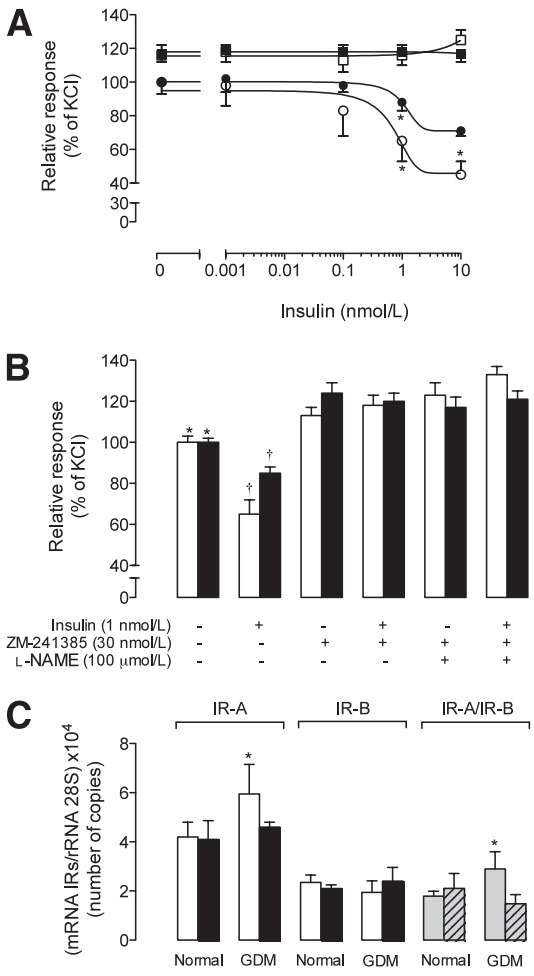

FIG. 6. Effect of insulin on human umbilical vein reactivity. *A*: Relaxation of 62.5 mmol/L KCl-preconstricted human umbilical vein rings isolated from normal (○, □) or GDM (●, ■) pregnancies incubated (8 h) without (0 nmol/L) or with insulin, in the absence (○, ●) or presence (□, ■) of L-NAME. *B*: 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.

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 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 supraphysiologic 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), A_{2A} and A_{2B} 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 A_{2A}-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 (IC_{50} GDM/IC_{50} 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 A_{2A}-adenosine receptor activation to cause vasodilatation because L-NAME and ZM-241385 abolished 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 A_{2B}-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

because the IR-A isoform predominates in diseases associated with insulin resistance, such as type 2 diabetic patients (33), and in states of insulin resistance associated with higher IR-A/IR-B ratio in skeletal muscles of patients with myotonic dystrophy types 1 (34) and 2 (34,35). In addition, along with the IR-A predominant expression in GDM, the reduced overall and hENT1-adenosine transport, hENT1 protein abundance, mRNA expression, and *SLC29A1* promoter activity were equally ($P > 0.05$) reduced (~60%) in GDM. Thus, these phenomena could result from IR-A overexpression in HUVEC from GDM.

Restoring IR-A mRNA expression in GDM to levels in normal pregnancies by insulin leads to recovery of hENT1-like transport activity, supporting the possibility that basal IR-A expression may be required to maintain normal adenosine transport activity. In addition, hENT1 protein abundance and mRNA expression in GDM cells were restored to values in normal cells, suggesting that insulin via IR-A 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 mitogenic 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, a phenomenon that could result in an elevated concentration 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 endothelium. Thus, it is feasible that a correction of the altered activity (and perhaps expression) of this type of nucleoside transporter could help to reduce the chance of the actual outcome of macrosomic newborns in GDM by limiting the resulting oversupply of metabolic substrates to the fetus.

Adenosine and insulin caused A_{2A}-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 adenosine transport and expression (including *SLC29A1* transcriptional 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 phenotype by insulin through modulation of extracellular adenosine levels and its A_{2A}-adenosine receptor–mediated biologic effects. In addition, because HUVECs from GDM preferentially express the IR-A isoform of insulin receptors (isoform associated with insulin resistance) (33–35) compared with cells from normal pregnancies, a crucial role for this type of insulin receptor in the fetoplacental vascular 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 research associate at CMPL-PRL); Pontificia Universidad Católica de Chile, Universidad de Antofagasta, Antofagasta, Chile; and Dirección de Investigación (DIUC 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 experimental 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 obtaining patients' clinical data; wrote, reviewed, and edited the manuscript; and drew the figures. F.C. contributed to generating experimental data. A.L. contributed to generating experimental data and obtaining patients' clinical data, wrote the manuscript, and drew the figures. P.C. contributed 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 Obstetrics and Gynecology, Faculty of Medicine, Pontificia Universidad Católica de Chile, for excellent secretarial assistance, and the personnel of the Hospital Clínico Pontificia Universidad Católica de Chile labor ward for supply of placentas.

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