Placenta 86 (2019) 35–44

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Neonates from women with pregestational maternal obesity show reduced umbilical vein endothelial response to insulin

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ARTICLE INFO

Keywords:
Obesity
Insulin resistance
Endothelium
Nitric oxide
Placenta

ABSTRACT

Objective: Pregestational maternal obesity (PGMO) associates with foetoplacental vascular endothelial dysfunction and higher risk for insulin resistance in the neonate. We characterised the PGMO consequences on the insulin response of the human foetoplacental vasculature.

Methods: Umbilical veins were from pregnancies where the mother was with PGMO (body mass index 30–42.3 kg/m^2, n = 33) or normal pregestational weight (PGMN) (body mass index 19.5–24.4 kg/m^2, n = 21) with total gestational weight gain within the physiological range. Umbilical vein ring segments were mounted in a myograph for isometric force measurements. Primary cultures of human umbilical vein endothelial cells were used in passage 3. Vessel rings and cells were exposed to 1 nmol/L insulin (20 min) in the absence or presence of 100 μmol/L N^G-nitro-L-arginine methyl ester (inhibitor of nitric oxide synthase, NOS).

Results: Vessel rings from PGMO showed reduced nitric oxide synthase-activity dependent dilation to insulin or calcitonin-gene related peptide compared with PGMN. PGMO associated with higher inhibitor phosphorylation of the insulin receptor substrate 1 (IRS-1) and lower activator phosphorylation of protein kinase B/Akt (Akt). Cells from PGMO also showed lower nitric oxide level and reduced activator serine^{1177} but increased inhibitor threonine^{495} phosphorylation of endothelial nitric oxide synthase (eNOS) and saturable transport of L-arginine. HUVECs from PGMO were not responsive to insulin.

Conclusion: The lack of response to insulin by the foetoplacental endothelium may result from reduced IRS-1/Akt/eNOS signalling in PGMO. These findings may result in higher risk of insulin resistance in neonates to PGMO pregnancies.

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https://doi.org/10.1016/j.placenta.2019.07.007
Received 2 April 2019; Received in revised form 9 July 2019; Accepted 15 July 2019

1. Introduction

Obesity in women in their reproductive age is a global problem (World Health Organization) [1] being a risk factor for complications of pregnancy [2–6]. As a consequence of this public health problem women show with body mass index (BMI) $\geq 30\,kg/m^2$ before pregnancy (i.e. pre-gestational maternal obesity, PGMO) [1] or at the first interview with their treating obstetrician, usually before the 12th weeks of gestation (see report from the Institute of Medicine (IOM) and the National Research Council (NRC)) [7]. PGMO is also a risk factor for infant and adolescent obesity [8,9] and associated with the development of metabolic syndrome later in life [10–12]. In addition, neonates from PGMO pregnancies show increased adiposity and insulin resistance [9,13–15], and long-term increased risk to develop insulin resistance [9,15–18].

Excessive (i.e. supra-physiological) gestational weight gain (spGWG) in women with normal pre-gestational BMI (18.5–24.9 kg/m^2) [1] associated with lower foetoplacental vascular dilation to insulin due to a reduced activity of the endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVECs) [19]. Also, insulin resistance associated with higher inhibitory phosphorylation of the insulin receptor substrate 1 (IRS-1) but lower protein kinase B/Akt (Akt), 44 and 42 kDa mitogen-activated protein kinases ($p44/42^{mapk}$), and eNOS activity in different types of endothelial and other cell types [9,20–25]. However, whether PGMO adversely influences the human foetoplacental endothelial function due to impaired vascular insulin signalling is unknown.

In this study, we characterised the response to insulin of the umbilical vein and primary cultured HUVECs from women with PGMO. The results suggest altered signalling in response to insulin which could lead to the reduced vascular dilation in PGMO.

2. Materials and methods

2.1. Study groups

Placenta with their umbilical cords were collected after delivery from 21 full-term pregnancies where the mother was with pre-gestational maternal normal weight (PGMN, BMI 18.5–24.9 kg/m^2) [1] or 33 full-term pregnancies where the mother was PGMO (BMI $\geq 30\,kg/m^2$) (see Supplementary Materials and methods). The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approvals from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and informed written consent of patients were obtained.

All pregnant women were evaluated for weight and height, and BMI was recorded at the first antenatal visit (i.e. 6–12 weeks of gestation) and delivery (37–40.5 weeks of gestation) (Table 1). Pregnant women with normal glycaemia at first trimester of pregnancy were also evaluated by an oral glucose tolerance test (OGTT) with a unique glucose load (75 g fasting) at 24–28 weeks of gestation to discard GDM (according to the Perinatal Guide 2015 from the Health Ministry of Chile) [26]. Pregnant women with PGMN or PGMO with diagnoses of GDM were not included in the study.

2.2. Maternal and neonatal blood samples

Plasma from umbilical venous blood collected immediately after birth was used for D-glucose (glucose oxidase method), insulin and C-peptide (immunoassay) levels determination. Neonatal insulin resistance index was calculated by the homeostasis model assessment for insulin resistance (HOMA-IR) [27] and insulin sensitivity by the quantitative insulin sensitivity check index (QUICKI) [28].

2.3. Human placenta and cell culture

Placentas with intact umbilical cords at delivery were disposed on ice and transferred to the laboratory within the next 15–30 min. Middle sections of umbilical cords were dissected into 200 mL phosphate-buffered saline (PBS) solution (mmol/L: 130 NaCl, 2.7 KCl, 0.8 Na2HPO4, 1.4 KH2PO4, pH 7.4, 4 °C) until use 6–12 h later. HUVECs were isolated by collagenase digestion (0.25 mg/mL Collagenase Type II from Clostridium histolyticum) (Boehringer, Mannheim, FRG) and cultured in primary culture medium (PCM: M199 containing 5 mmol/L D-glucose, 10% newborn calf serum (NBCS), 10% foetal calf serum (FCS), 3.2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin (Gibco Life Technologies, Carlsbad, CA, USA)) under standard conditions [29]. Experiments were in the absence ('without insulin') or presence (20 min, 'with insulin') of regular (rapid-acting) insulin (1 nmol/L) (Humulin-R, 100 IU/mL) (Eli Lilly and Company, Indianapolis, IN, USA). Cells were also exposed (30 min) to N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L, NOS inhibitor) [30] and sodium nitroprusside dehydrate (SNP, 100 μmol/L, spontaneous NO donor) (Sigma Aldrich, St Louis, MO, USA) [31].

2.4. Umbilical vein reactivity

Ring segments (2–4 mm length) dissected from human umbilical cord veins were mounted in a myograph (610 M Multiwire Myograph System, Danish Myo Technology A/S, Denmark) for isometric force measurements in Krebs solution (mmol/L: 118.5 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 5.5 D-glucose, 0.3 L-arginine, 37 °C, pH 7.4) as described [19,23]. The optimal diameter for vessels was adjusted by determining the maximal active response evoked by 65 mmol/l KCl [19,23]. Endothelium-dependent relaxation was evaluated as the concentration-dependent response to calcitonin gene related protein (CGRP, 0.01–100 nmol/L, 5 min) (Sigma Aldrich) and insulin (0.1–1000 nmol/L, 5 min) in 32.5 mmol/L KCl-pre-constricted vessels. Changes in isometric tension were recorded using the software LabChart 7 for Windows coupled to a PowerLab 8/30 Data Acquisition System (ADInstruments, Australia).

2.5. Insulin receptor β-subunit immunoprecipitation

Confluent HUVECs were lysed, sonicated, and centrifuged for protein separation as described [32]. Insulin receptor β-subunit (β-IR) was assayed using a monoclonal mouse *anti*-β-IR antibody (1:5000 dilution) (Sigma-Aldrich) and separated by polyacrylamide gel (10%) electrophoresis (PAGE) as described [32].

2.6. Western blotting

Total proteins (50 μg) were separated by 10% PAGE and probed against total and $serine^{473}$ phosphorylated Akt, total and $threonine^{202}$/$tyrosine^{204}$ phosphorylated $p44/42^{mapk}$, total and $serine^{1177}$ or $threonine^{495}$ phosphorylated eNOS, total and $serine^{307}$ phosphorylated IRS-1, total and $tyrosine^{1361}$ phosphorylated β-IR, and β-actin. Proteins detected by enhanced chemiluminescence were quantified by densitometry as described [29].

2.7. Intracellular NO determination

The NO level was measured in HUVECs loaded (5 μmol/L, 1 h) with the fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) (Merck Millipore, Billerica, MA, USA) in the absence or presence of L-NAME (100 μmol/L, 30 min) in M199 without phenol red (Gibco Life Technologies, Carlsbad, CA, USA) as described [23,29]. Fluorescence was measured in a fluorescent Tecan Infinite M200 PRO microplate reader (Tecan, Untersbergstr, Austria) at 37 °C and calibrated for excitation/emission at 485/538 nm.

Table 1
Clinical characteristics of pregnant women and neonates.

	PGMN (n = 21)	PGMO (n = 33)
Maternal variables		
Age (years)	29.3 ± 5.3 (21–40)	31.3 ± 3.9 (24–38)
Height (cm)	161.0 ± 5.3 (149–170)	161.4 ± 7.9 (140–175)
Weight (kg)		
3–12 wg	58.0 ± 1.2 (50–64)	84.1 ± 3.4 (69.3–93.7) *
37–40 wg	70.1 ± 1.4 (61–83) †	92.5 ± 4.3 (80.0–101.0) *†
BMI (kg/m^2)		
3–12 wg	22.4 ± 1.3 (19.5–24.4)	33.0 ± 3.9 (30.0–42.3) *
37–40 wg	27.0 ± 1.3 (24.0–28.7)	36.3 ± 5.2 (32.1–46.7) *
Gestational weight gain (kg)	14.3 ± 3.3 (12–15.9)	8.4 ± 2.9 (5.1–9)
OGTT (mmol/L)		
Glycaemia basal	4.4 ± 0.5 (3.7–5.5)	4.4 ± 0.3 (3.7–4.9)
Glycaemia 2 h after glucose load	6.0 ± 0.7 (5.2–7.1)	6.1 ± 1.3 (4.0–8.8)
Neonate variables		
Sex (female/male)	10/11	14/19
Gestational age (weeks)	39.3 ± 0.82 (38–40.5)	39.0 ± 0.83 (37–40.5)
Weight (grams)	3451 ± 307 (3000–4150)	3495 ± 403 (2710–4400)
Height (cm)	50.8 ± 1.95 (47–54)	50.9 ± 1.63 (47.5–54)
Ponderal index (grams/cm^3 x 100)	2.65 ± 0.25 (2.22–3.24)	2.64 ± 0.21 (2.30–3.26)
Umbilical vein glycaemia (mmol/L)	3.4 ± 0.1 (2.7–4.1)	3.7 ± 0.1 (3.2–4.3)
Umbilical vein insulin (μU/mL)	5.01 ± 0.36 (2.6–6.4)	8.36 ± 1.78 (4.6–19.7) *
Umbilical vein C-peptide (ng/mL)	0.65 ± 0.03 (0.50–0.90)	0.94 ± 0.12 (0.58–1.50) *
HOMA-IR	0.44 ± 0.02 (0.37–0.57)	1.05 ± 0.28 (0.52–2.64) *
QUICKI	0.40 ± 0.02 (0.36–0.47)	0.36 ± 0.04 (0.31–0.41) *

PGMN, pregestational maternal normal weight; PGMO, pregestational maternal obesity; wg, weeks of gestation; BMI, body mass index; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment for insulin resistance; QUICKI, quantitative insulin sensitivity check index. OGTT was measured between 24 and 28 wg. HOMA-IR was calculated from IR = $\frac{\text{Insulin} \cdot \text{Glucose}}{22.5}$ where Insulin is in μU/mL and Glucose is basal glycaemia in mmol/L as described [27]. QUICKI was calculated from QUICKI = $\frac{1}{(\log \text{Insulin} + \log \text{Glucose})}$ with Insulin and Glucose as in HOMA-IR as described [28]. *$P < 0.05$ versus corresponding values in PGMN. †$P < 0.05$ versus corresponding values at 3–12 wg. Values are mean ± S.D. (range).

2.8. L-Arginine transport

Overall L-arginine transport (3 μCi/mL L-[^{3}H]arginine (NEN (Dreieich, FRG), 1 min, 37 °C) was measured in Krebs solution (mmol/L: 131 NaCl, 5.6 KCl, 25 $NaHCO_3$, 1 NaH_2PO_4, 20 Hepes, 2.5 $CaCl_2$, 1 $MgCl_2$, pH 7.4, 37 °C) as described [23,29]. Overall transport resulted from the sum of a saturable component plus a nonsaturable, linear component of transport (K_D) defined by m•[Arg], where m is slopes of linear phases of transport at a given L-arginine concentration [Arg]. The initial transport rate was derived from the slope of the linear phases of L-arginine transport. Values for transport were adjusted to the one phase exponential association equation considering the least squares fit:

$$v_i = V_m \cdot (1 - e^{-(k \cdot t)})$$

where v_i is initial velocity, V_m is mayor velocity at a given time (t) and L-arginine concentration, and e and k are constants. Overall L-arginine transport at initial rates was adjusted to the Michaelis-Menten hyperbola plus a nonsaturable, linear component [23,29]. The saturable transport of L-arginine was derived by subtracting the m•[Arg] components from overall transport, and the transport kinetic parameters maximal velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) of transport were calculated. The relative contribution of insulin to the saturable L-arginine transport kinetic parameters was estimated from the maximal transport capacity (V_{max}/K_m) values for L-arginine transport by:

$$\frac{1}{{}^{C/Ins}F} = \frac{{}^{C}Km \cdot {}^{Ins}Vmax}{{}^{C}Vmax \cdot {}^{Ins}Km}$$

where ${}^{C}V_{max}$ and ${}^{C}K_m$ are the kinetics parameters for L-arginine transport in control conditions (i.e., in the absence of insulin), and ${}^{Ins}V_{max}$ and ${}^{Ins}K_m$ are kinetics parameters of L-arginine transport in the presence of insulin [23].

2.9. Statistical analysis

The sample size was estimated considering a power of 80% enough to detect differences between both groups (based on a two-sided alpha level of 0.05). Values for clinical parameters are as mean ± S.D. (range). For *in vitro* assays the values are mean ± S.E.M., where n indicates the number of different biological placentas and corresponding cell cultures (n = 18 per group) or umbilical vein rings (n = 7 per group) with 2–3 replicates per experiment. Comparisons between two groups were performed using Student's unpaired t-test or Mann-Whitney test for parametric or non-parametric data, respectively. The normality of the data (i.e. parametric) was confirmed with the D'Agostino-Pearson omnibus test. The variances across groups under Bartlett's test were homogeneous. The difference between more than two groups were performed by analysis of variance (ANOVA, one or two-ways). If the ANOVA demonstrated a significant interaction between variables, *post hoc* analyses were performed by the multiple-comparison Tukey test. The statistical software GraphPad Instat 3.0b and GraphPad Prism 8.1.0 (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. $P < 0.05$ was considered significant.

3. Results

3.1. Pregnant women and neonates

Women were of comparable age and height and similar OGTT and gestational weight gain (GWG) from 6 to 12 weeks of gestation up to delivery (Table 1). Women with PGMO showed higher weight at the beginning of pregnancy than PGMN. Both groups showed an increase in total GWG within the recommended ranges for pregnant women with normal weight (11.5–16 kg) or obesity (5–9 kg) [7]. Gestational age, birth weight and height, ponderal index, and umbilical vein glycaemia at birth were comparable between the neonates from both groups. Neonates from PGMO had greater umbilical vein blood level of insulin

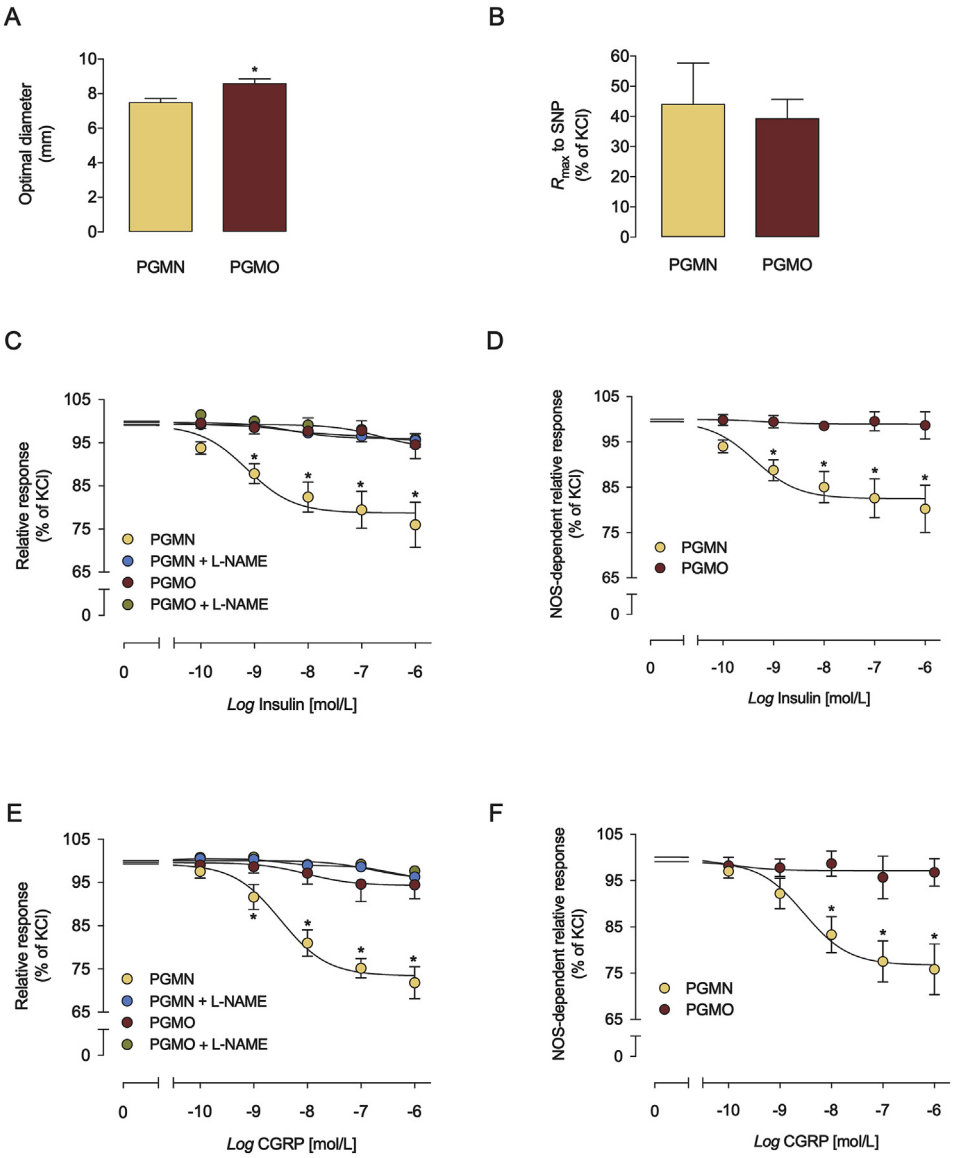

Fig. 1. Dilation of human umbilical vein rings. A. Human umbilical vein rings were isolated from pregnancies where the mother showed pre-gestational maternal normal weight (PGMN) or pre-gestational maternal obesity (PGMO). Vessel rings were mounted in a myograph for isometric force measurements. The optimal diameter for each vessel was adjusted through the determination of the maximal active response evoked by 65 mmol/L KCl. (see Materials and methods). B. Maximal relaxation (R_{max}) of umbilical vessel rings in response to 1 nmol/L sodium nitroprusside dehydrate (SNP, 100 μmol/L). C. Human umbilical vein rings from PGMN or PGMO pre-constricted with 32.5 mmol/L KCl were incubated without (0) or with insulin (5 min) in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L, 30 min). D. Nitric oxide synthase (NOS) activity-dependent relative response of vein rings to insulin derived from data in C. E. Vein rings response to calcitonin gene-related protein (CGRP, 5 min) as in C. Relative responses are as a percentage of the fraction of the initial vessel response to KCl (see Materials and methods). F. Nitric oxide synthase (NOS) activity-dependent relative response of vein rings to CGRP derived from data in E. In A, $^*P < 0.05$ versus PGMN. In C-F, $^*P < 0.05$ versus all other values for the corresponding concentrations of insulin or CGRP. Values are mean ± S.E.M. ($n = 7$).

(1.67 ± 0.28 fold) and C-peptide (1.45 ± 0.17 fold), increased HOMA-IR (2.39 ± 0.31 fold) and lower sensitivity to insulin (10 ± 2%) compared with PGMN pregnancies.

3.2. Umbilical vein reactivity

To determine whether PGMO shows altered umbilical vein reactivity the insulin-induced dilation of umbilical vein rings was assayed. Vein rings from PGMO showed higher (1.15 ± 0.02 fold) optimal diameter compared with vessels from PGMN pregnancies (Fig. 1A). SNP caused similar dilation in vein rings from both groups of patients (Fig. 1B). Insulin caused a concentration-dependent dilation of vein rings from PGMN (half maximal vasodilatory effect (EC_{50}) = 0.73 ± 0.06 nmol/L) with R_{max} = 21.3 ± 1.5% at 1 μmol/L. However, the response of vein rings from PGMO to insulin was almost absent (R_{max} = 5.4 ± 3.5% at 1 μmol/L) (Fig. 1C). L-NAME prevented the relaxation to insulin in PGMN (R_{max} = 4.1 ± 0.3% at 1 μmol/L) and the NOS activity-dependent response to insulin showed lower EC_{50} (0.42 ± 0.06 nmol/L; $P < 0.05$) but similar R_{max} (17.0 ± 1.8%) compared insulin effect in absence of L-NAME (Fig. 1D). Dilation to CGRP showed a similar pattern to insulin in PGMN or PGMO (Fig. 1E and F). Dilation caused by CGRP in vein rings from PGMN showed higher EC_{50} (3.17 ± 0.01 nmol/L, $P < 0.05$) but similar R_{max} (25.9 ± 1.1% at 1 μmol/L) compared with insulin response (Fig. 1E). L-NAME prevented the dilation to CGRP in PGMN (R_{max} = 4.3 ± 0.9%) and NOS activity-dependent CGRP dilation showed higher EC_{50} (2.96 ± 0.14 nmol/L, $P < 0.05$) but similar R_{max} (22.4 ± 0.2%) compared with the response to insulin (Fig. 1F).

3.3. β-IR and IRS-1 phosphorylation

To determine whether the reduced dilation to insulin in vein rings from PGMO resulted from altered insulin signalling, activator and inhibitor phosphorylation of proteins involved in the endothelial insulin/NO signalling pathway was assayed [23,29]. Phosphorylated and total β-IR protein abundance in the absence of insulin were similar in cells from PGMN and PGMO pregnancies (Fig. 2A). Insulin caused comparable increase in phosphorylated β-IR in PGMN (9.6 ± 3.1 fold) and PGMO (10.1 ± 2.9 fold), without altering the total β-IR protein abundance. Total IRS-1 protein abundance was similar in the absence or presence of insulin in PGMN and PGMO (Fig. 2B). HUVECs from PGMO showed higher (3.9 ± 1.1 fold) inhibitor Ser^{307} phosphorylation of IRS-1 in the absence of insulin compared with PGMN, an effect unaltered by insulin in both groups (Fig. 2B).

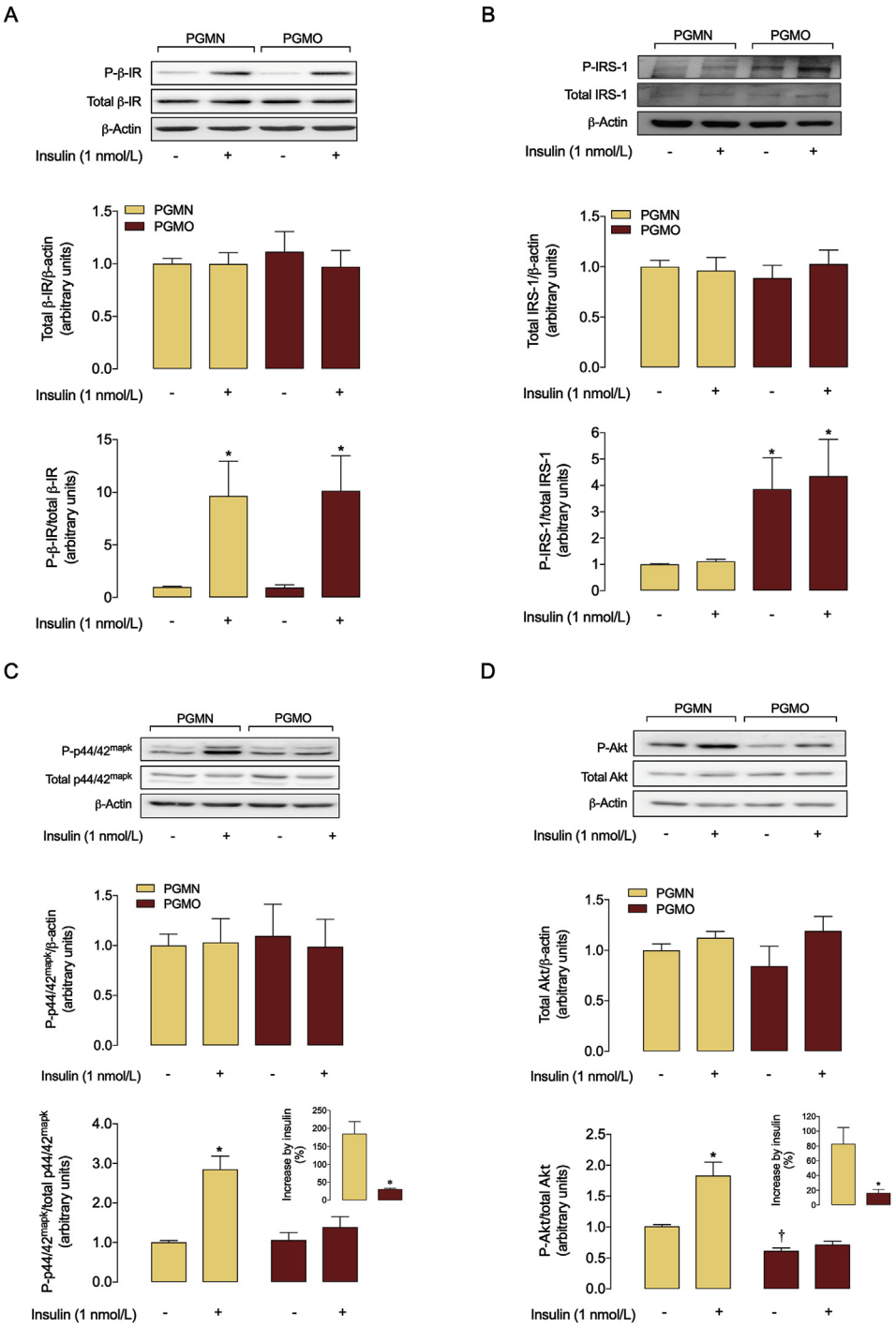

Fig. 2. Insulin modulation of β-IR subunit, IRS-1, p44/42^{mapk} and Akt expression in HUVECs. A. Western blot for total β-IR (Total β-IR), phosphorylated β-IR (P-β-IR), and β-actin (internal reference) protein abundance in HUVECs from pregnancies where the mother showed pre-gestational maternal normal weight (PGMN) or pre-gestational maternal obesity (PGMO). Cells were incubated without (–) or with (+) insulin (20 min). *Lower panels*: Total β-IR/β-actin or P-β-IR/total β-IR ratio densitometries normalised to 1 in PGMN without insulin. B. Western blot for total IRS-1 (Total IRS-1), phosphorylated IRS-1 (P-IRS-1), and β-actin protein abundance as in A. *Lower panels*: Total IRS-1/β-actin or P-IRS-1/total IRS-1 ratio densitometries normalised to 1 in PGMN without insulin. C. Western blot for total p44/42^{mapk} (Total p44/42^{mapk}) and phosphorylated p44/42^{mapk} (P-p44/42^{mapk}), and β-actin (internal reference) protein abundance as in A. *Lower panels*: Total p44/42^{mapk}/β-actin or P-p44/42^{mapk}/total p44/42^{mapk} ratio densitometries normalised to 1 in PGMN without insulin. The *insert* shows the increase caused by insulin in cells from PGMN or PGMO expressed as percent of the corresponding values in absence of insulin. D. Western blot for total Akt (Total Akt) and phosphorylated Akt (P-Akt), and β-actin (internal reference) protein abundance as in A. *Lower panels*: Total Akt/β-actin or P-Akt/total Akt ratio densitometries normalised to 1 in PGMN without insulin. *Inserts* in C and D show the increase caused by insulin in cells from PGMN or PGMO expressed as percent of the corresponding values in absence of insulin. In A, $^{*}P < 0.04$ compared with corresponding values without insulin. In B, $^{*}P < 0.04$ versus corresponding values in PGMN. In C, $^{*}P < 0.05$ versus all other values. *Insert*: $^{*}P < 0.04$ versus PGMN. In D, $^{*}P < 0.05$ versus all other values, †$P < 0.05$ versus PGMN without insulin, *Insert*: $^{*}P < 0.04$ versus PGMN. Values are mean ± S.E.M. ($n = 18$).

3.4. p44/42^{mapk} and Akt phosphorylation

Activation of β-IR and IRS-1 by insulin triggers phosphorylation of p44/42^{mapk} and Akt in HUVECs [9,24,32], therefore, the state of p44/42^{mapk} and Akt phosphorylation was determined. Total p44/42^{mapk} (Fig. 2C) and Akt (Fig. 2D) protein abundance was similar in cells from PGMN and PGMO in the absence or presence of insulin. In the absence of insulin, PGMO did not alter p44/42^{mapk} phosphorylation compared with PGMN. Insulin increased the phosphorylation of p44/42^{mapk} in cells from PGMN (2.8 ± 0.4 fold) but not from PGMO. However, PGMO associated with lower Akt phosphorylation compared with PGMN. Insulin increased the Akt phosphorylation in cells from PGMN but did not alter the PGMO-associated reduction in Akt phosphorylation.

3.5. eNOS expression and NO synthesis

Insulin increases NO synthesis in HUVECs and causes NO-dependent dilation of human umbilical vein rings [19,23]. Thus, eNOS expression and activity was evaluated. Total eNOS protein abundance was similar in cells from PGMO and PGMN pregnancies and was unaltered by insulin (Fig. 3A). In the absence of insulin, eNOS phosphorylation at Ser^{1177} was lower (36 ± 4%) (Fig. 3B), but phosphorylation at Thr^{495} was higher (2.1 ± 0.6 fold) (Fig. 3C) in PGMO compared with PGMN. Insulin increased the Ser^{1177} phosphorylation (2.2 ± 0.4 fold) in cells from PGMN but did not alter the reduced Ser^{1177} phosphorylation seen

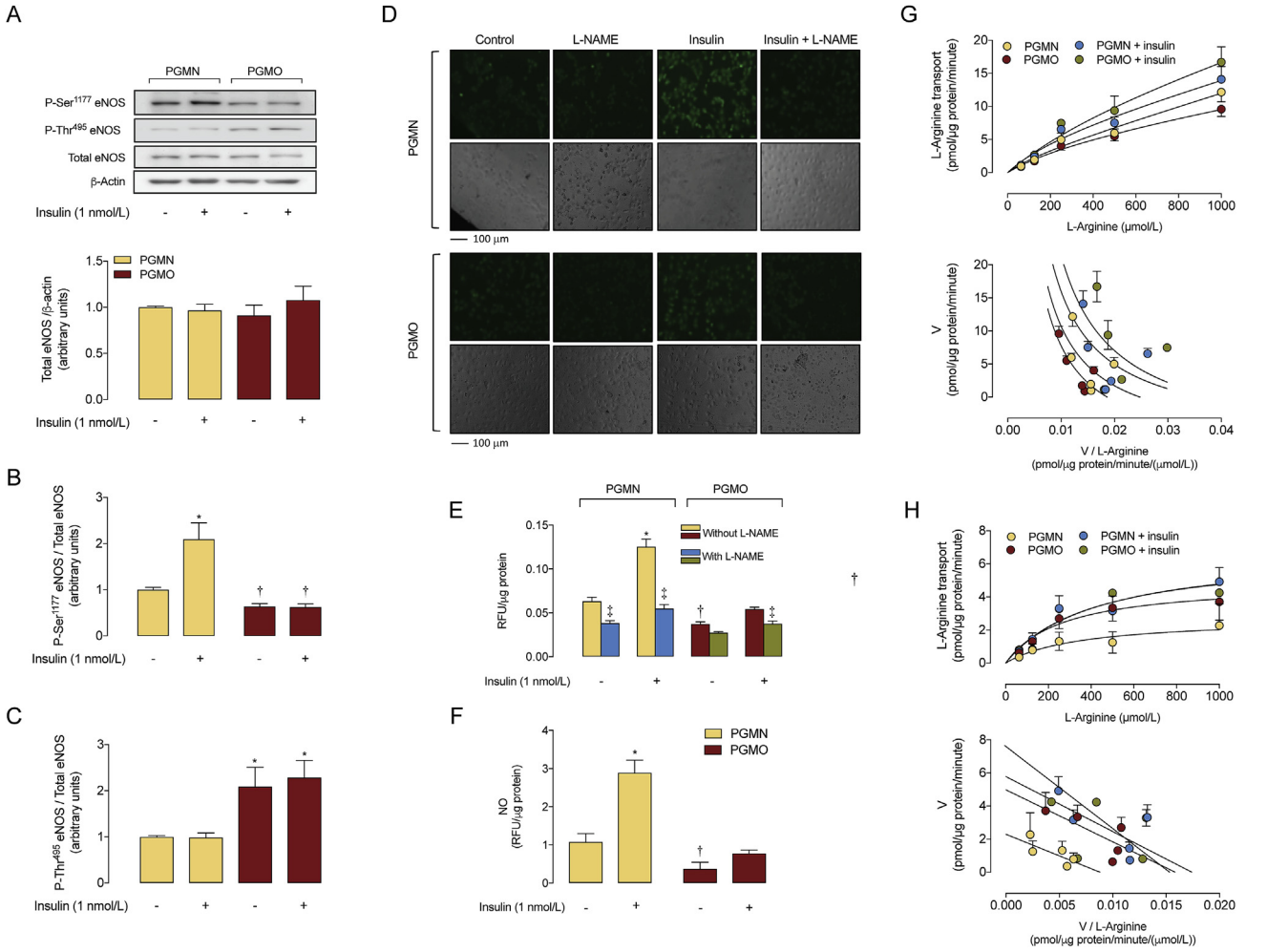

Fig. 3. Insulin modulation of eNOS and L-arginine transport in HUVECs. A. Western blot for total eNOS (Total eNOS) and phosphorylated eNOS in serine[1177] (P-Ser[1177] eNOS) or threonine[495] (P-Thr[495] eNOS), and β-actin (internal reference) protein abundance in HUVECs from pregnancies where the mother showed pre-gestational maternal normal weight (PGMN) or pre-gestational maternal obesity (PGMO). Cells were incubated without (–) or with (+) insulin (20 min). *Lower panels*: Total eNOS/β-actin (A), P-Ser[1177] eNOS/total eNOS (B), and P-Thr[495] eNOS/total eNOS (C) ratio densitometries normalized to 1 in PGMN without insulin. D. Fluorescence in cells preloaded with 4-amino-5-methylamino-2,7-difluorescein (10 μmol/L) (see Materials and methods). Fluorescence signal for nitric oxide was assayed in HUVECs incubated in the absence (Control) or presence of 1 nmol/L insulin (20 min) without or with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L, 30 min). Images are representative of triplicates from seven different cell cultures (magnification x40). E. Total relative fluorescence units (RFU) in cells as in D. F. Nitric oxide (NO) level obtained from the total RFU corresponding to the fraction inhibited by L-NAME from data in E. G. Overall transport of L-arginine (1 min, 37 °C, 6 μCi/mL L-[^{3}H]arginine) in HUVECs incubated without or with insulin (1 nmol/L, 20 min) as in A. Data were adjusted to the Michaelis-Menten hyperbola plus a nonsaturable, linear component (see Materials and methods). *Lower panel*: Eadie-Hofstee plots for overall transport data. H. Saturable transport of L-arginine derived from data in G adjusted to a single Michaelis-Menten equation. *Lower panel*: Eadie-Hofstee plots for saturable transport data. In B, *$P < 0.05$ versus all other values, †$P < 0.05$ versus PGMN without insulin. In C, *$P < 0.05$ versus corresponding values in PGMN. In E, *$P < 0.05$ all other values, †$P < 0.05$ versus PGMN without insulin and L-NAME, and PGMO with insulin without L-NAME, ‡$P < 0.05$ versus corresponding values without L-NAME. In F, *$P < 0.05$ all other values, †$P < 0.05$ versus PGMN without insulin. Values are mean ± S.E.M. ($n = 12$).

in PGMO. However, incubation of cells with insulin did not alter Thr[495] phosphorylation in HUVECs from both study groups.

To confirm that PGMO or insulin modify NOS activity, the NOS activity-dependent NO level was measured. Fluorescence for DAF-FM probe was seen in cells from both study groups (Fig. 3D). Fluorescence in the absence of insulin and L-NAME was lower (41 ± 6%) in cells from PGMO compared with PGMN pregnancies (Fig. 3E). L-NAME reduced the fluorescence in cells from PGMN but not from PGMO. Insulin increased the fluorescence in cells from both conditions, a response that was blocked by L-NAME. The fluorescence detected in cells pre-incubated with L-NAME in the absence of insulin (i.e. basal level of NO generated from NOS activity) was lower (62 ± 8%) in cells from PGMO compared with PGMN (Fig. 3F). Insulin increased (2.8 ± 0.4 fold) the NO level in cells from PGMN pregnancies but did not alter the PGMO-reduced NO level.

3.6. L-Arginine transport

L-Arginine transport is shown to parallel NO synthesis compensating the lack of NO bioavailability in human endothelial cells [19,33,34]. Therefore, we measured L-arginine transport in cells from PGMO in the absence or presence of insulin. The v_i for overall 100 μmol/L L-arginine transport was linear up to 60 s in cells from PGMN and PGMO in the absence or presence of insulin (not shown). The v_i was higher in cells from PGMO compared to PGMN (Table 2). Insulin increased the v_i in PGMN to values similar to those in PGMO but did not restore the PGMO-associated increase in this parameter.

Overall L-arginine transport in the absence or presence of insulin was semi-saturable and best-fitted by a non-linear representation in Eadie-Hofstee plots in cells from PGMN and PGMO (Fig. 3G). The K_D values were unaltered by PGMO or in response to insulin compared

Table 2
Kinetic parameters for L-arginine transport in HUVECs from PGMN or PGMO.

	Saturable transport		*Overall transport*		
	V_{max} (pmol/μg protein/minute)	K_m (μmol/L)	V_{max}/K_m (pmol/μg protein/minute/(μmol/L))	K_D (pmol/μg protein/minute/(μmol/L))	v_i (pmol/μg protein/0.5 s)
PGMN	2.74 ± 0.35	356 ± 97	0.0067 ± 0.0241	0.00988 ± 0.0043	0.00228 ± 0.00020
PGMN + insulin	6.04 ± 0.74 *	268 ± 37	0.0225 ± 0.0029 *	0.00941 ± 0.0035	0.00503 ± 0.00011 *
PGMO	4.94 ± 0.29 *	272 ± 96	0.0182 ± 0.0038 *	0.00588 ± 0.0062	0.00333 ± 0.00012 *
PGMO + insulin	7.07 ± 1.25 *	371 ± 140	0.0191 ± 0.0053 *	0.00998 ± 0.0024	0.00589 ± 0.00008 *

L-Arginine transport (0–1000 μmo/L, 1 min, 37 °C) was measured in HUVECs from women with pre-gestational normal weight (PGMN) or obesity (PGMO). Cells were exposed to culture medium without or with insulin (1 nmol/L, 20 min) (see Materials and methods). Maximal velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) of saturable transport were calculated assuming a single Michaelis-Menten hyperbola. The V_{max}/K_m represents maximal L-arginine transport capacity. The lineal phase of overall transport of L-arginine (K_D) was obtained from transport data fitted to a Michaelis-Menten equation increased in a lineal component. Initial velocity (v_i) was calculated for 0.5 s with 100 μmol/L L-arginine transport. *$P < 0.05$ versus values in PGMN. Values are mean ± S.E.M. ($n = 12$).

with PGMN (Table 2). After subtracting the linear, non-saturable transport component from the overall transport, the remaining L-arginine transport was saturable, adjusted to a single Michaelis-Menten equation, and linear in Eadie-Hofstee plots (Fig. 3H). Saturable transport in PGMO showed higher V_{max} and maximal transport capacity (V_{max}/K_m) compared with cells from PGMN pregnancies (Table 2). In cells from PGMN pregnancies, the V_{max} and V_{max}/K_m were increased by insulin; however, PGMO-associated increase in V_{max} and V_{max}/K_m was unaltered by this hormone. The apparent K_m values were not significantly altered in cells from these two study groups in the absence or presence of insulin.

4. Discussion

This study addresses that human umbilical vein rings from women with PGMO show almost no response to insulin compared with PGMN pregnancies. This phenomenon may result from IRS-1 inhibition, reduced Akt activation and NO generation by the umbilical vein endothelium. These results suggest defective modulation of the foetoplacental vascular reactivity to insulin in pregnancies where the mother showed pre-pregnancy obesity.

Neonates to PGMO show higher umbilical vein blood levels of insulin and C-peptide, agreeing with the reported positive correlation between changes in these parameters and fat mass in neonates to mothers with obesity [13]. Since increased C-peptide level associated with insulin resistance in preterm infants [35,36], the higher C-peptide and insulin values in the umbilical blood in PGMO suggest lower neonate insulin sensitivity. This is supported by the results showing that calculated QUICKI [28] was reduced in ~10% and HOMA-IR [27] was ~2.4 fold higher in neonates from PGMO. Thus, a potential state of insulin resistance in these individuals is likely. PGMO associated with higher risk of spGWG and negative pregnancy outcomes including macrosomia, caesarean delivery, maternal complications, metabolic syndrome, and cardiovascular disease in adulthood [11,37–40]. Since women included in this study showed total GWG (PGMN: 12.1 kg, PGMO: 8.4 kg) and rates of weight gain (PGMN: 0.39 kg/week, PGMO: 0.25 kg/week) within the IOM/NRC recommended values for pregnant women [7], a potential involvement of spGWG in the results is unlikely.

4.1. Foetoplacental vascular dysfunction

PGMO associated with lack of response of umbilical vein rings to insulin. This phenomenon may result from a defective vascular endothelium rather than vascular smooth muscle since the vascular response to SNP, a NO donor, remained intact in these vessels. Also, NOS activity-dependent dilation to insulin was almost absent and the response to CGRP, a vasodilator that preferentially activates the endothelium [41], is largely reduced (~94%) in PGMO. Interestingly, the required insulin to cause NOS-dependent compared with total dilation (i.e. NOS-dependent plus other mechanisms) of vein rings from PGMN [1 – ($^{NOS}EC_{50}/R_{max}$)/($^{Total}EC_{50}/R_{max}$)] was ~30% lower suggesting a larger involvement of NOS activity in the response of human vein rings to this hormone. NOS-dependent dilation was entirely endothelium dependent since the CGRP required to cause total and NOS-dependent dilation was similar [1 –($^{NOS}EC_{50}/R_{max}$)/($^{Total}EC_{50}/R_{max}$) ~1.08]. HUVECs are activated by acetylcholine resulting in increased eNOS activity [42]. However, whether a potential cholinergic response of umbilical veins is altered in pregnancies where the mother was with PGMO is unknown.

4.2. Insulin signalling

Insulin activates insulin receptor A (IR-A) and IR-B leading to β-IR autophosphorylation [9,43,44]. The results show that insulin-increased β-IR phosphorylation was similar in HUVECs from PGMO and PGMN. Thus, PGMO-associated lack of response to insulin does not result from reduced insulin receptors autophosphorylation. IR-A activation results in preferential recruitment and activation of IRS-1 in the human foetoplacental vasculature [9,45]. Since the inhibitory IRS-1 Ser^{307} phosphorylation was higher in PGMO, this metabolic condition may result in IRS-1 inhibition. The latter agrees with the increased IRS-1 Ser^{307} phosphorylation seen in skeletal muscle from obese individuals [46] and in animal models of obesity-dependent insulin resistance [47,48]. Therefore, reduced IRS-1 activation may explain the lower response to insulin in vein rings from PGMO. The finding that IRS-1 Ser^{307} phosphorylation was unaltered by insulin in HUVECs from PGMO may be due to a maximal induction of this post-translational modification of IRS-1.

Inhibitory IRS-1 Ser^{307} phosphorylation in HUVECs from PGMO may reduce insulin-triggered $p44/42^{mapk}$ and Akt-mediated signalling in foetal endothelium as in most cells [9,15,20,49]. Since basal phosphorylation of Akt, but not $p44/42^{mapk}$, in HUVECs from PGMO was lower compared with PGMN, a defective Akt-signalling in PGMO is likely. Interestingly, reduced Akt phosphorylation in PGMO was unaltered by insulin. Thus, the lack of response of HUVECs from PGMO to insulin may result from deficient Akt activation. Since IR-B signals preferentially via Akt compared with IR-A preferential signalling through $p44/42^{mapk}$ activation (i.e. $p44/42^{mapk}$/Akt < 1) in HUVECs [23,32], PGMO may have reduced insulin-triggered IR-B/Akt signalling in this cell type.

HUVECs from PGMO showed lower basal NO level compared with PGMN. This phenomenon may result from reduced eNOS activity since activator eNOS Ser^{1177} phosphorylation was lower and inhibitor eNOS Thr^{495} phosphorylation was higher in PGMO. Akt activation results in eNOS activation in HUVECs [9,22,23,25,30]. Because PGMO reduced Akt activation and increased IRS-1 inhibition, a lower IRS-1/Akt signalling may have resulted in a lower basal NO synthesis in HUVECs. On the other hand, $p44/42^{mapk}$ activity is unaltered in PGMO, making

unlikely a role for these kinases in the lack of response to insulin in vein rings and HUVECs. However, the role of p44/42^{mapk} as modulator of eNOS activity in HUVECs [50,51] and other endothelial cells [52,53] is still unclear.

It is reported that higher L-arginine uptake, the NOS substrate [30], activated eNOS in HUVECs [33]. PGMO associated with higher maximal transport capacity (V_{max}/K_m) due to increased V_{max} compared with PGMN pregnancies. Because the saturable component of L-arginine transport was linear in Eadie-Hofstee plots, either a single transport system or two or more transport systems with similar apparent K_m may be involved in this phenomenon. Apparent K_m values in this study are within the range of values described for hCAT-1 (~100–250 μmol/L) and hCAT-2B (~200–400 μmol/L) in HUVECs [23,33]. Thus, both membrane transporters may account for the increased L-arginine transport in this cell type from PGMO. This possibility complemented a similar increase in hCAT-1–mediated L-arginine transport characterized in HUVECs from women with PGMO and physiological GWG [29]. Increased L-arginine transport in cells showing reduced eNOS activity in PGMO may result as a response to the low NO bioavailability in these cells [29]. The latter agrees with similar phenomena in response to intracellular alkalization [33] and cells challenged with HUVECs-derived exosomes from women with GDM [54]. Insulin did not alter the V_{max}/K_m for L-arginine transport in cells from PGMO but increased this parameter ($1/^{C/Ins}F$ ~3.5 fold) in PGMN. This phenomenon may explain why insulin is ineffective in restoring the reduced NO generation seen in cells from PGMO.

In summary, PGMO is an abnormal metabolic condition associated with a neonate's subclinical state of insulin resistance. This possibility is supported by the reduced reactivity to insulin in umbilical vein rings from PGMO likely due to a defective insulin signalling via IRS-1/Akt but unaltered autophosphorylation of insulin receptors (Fig. 4). These alterations lead to lower eNOS activation which may be a factor increasing the L-arginine transport via hCAT1 and hCAT-2B isoforms as an adaptive response in HUVECs from PGMO. We speculate that a defective insulin signalling in HUVECs from PGMO pregnancies is a condition that could lead to higher risk of insulin resistance-associated metabolic disorders of the neonates and in their young and adulthood.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Author contributions

Roberto Villalobos-Labra: I declare that I participated in the development of the original idea, performed experiments, analysed the literature and results, drafted the manuscript, made a critical reading of the document, generate the first draft of the figures, and directly contribute to the discussion. I have seen and approved the final version.

Francisco Westermeier: I declare that I participated in performing initial experiments, analysed the literature and results and made a critical reading of the manuscript. I have seen and approved the final version.

Carolina Pizarro: I declare that I participated in performing initial experiments, analysed the literature and results and made a critical reading of the manuscript. I have seen and approved the final version.

Pablo J Sáez: I declare that I participated in performing initial experiments, analysed the literature and results and made a critical reading of the manuscript. I have seen and approved the final version.

Fernando Toledo: I declare that I participated in analysing the results and statistics and made a critical reading of the manuscript. I have seen and approved the final version.

Fabián Pardo: I declare that I participated in analysing the results and made a critical reading of the manuscript. I have seen and approved the final version.

Juan P Kusanovic: I declare that I participated in making a critical reading of the manuscript. I have seen and approved the final version.

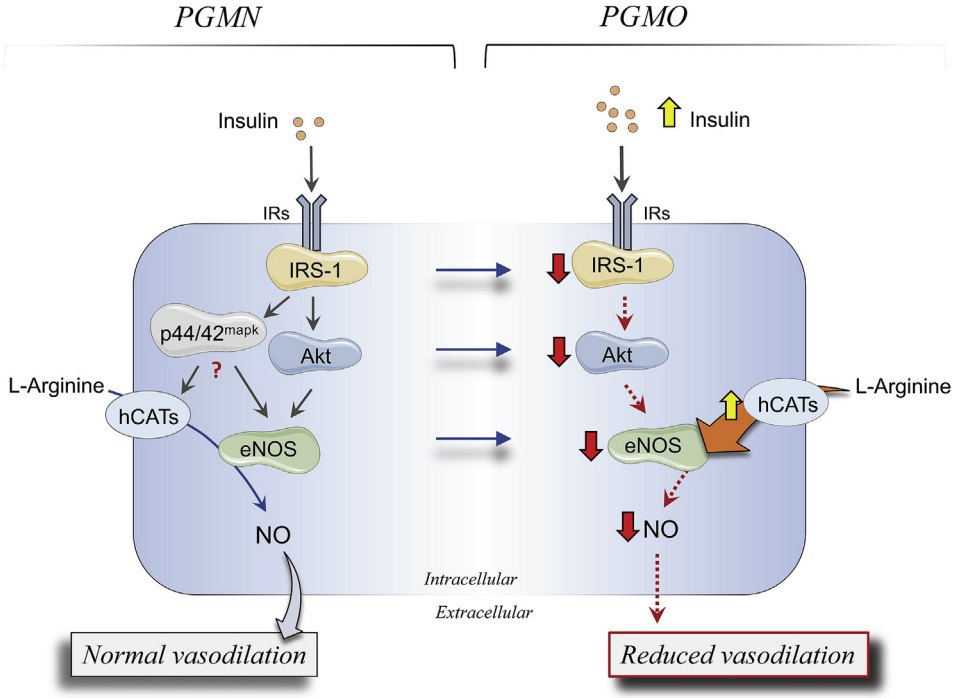

Fig. 4. Reduced relaxation to insulin in human umbilical vein from pre-gestational maternal obesity. In human umbilical vein endothelial cells (HUVECs) from pregnancies where the mother was with pre-gestational maternal normal weight (*PGMN*), insulin activates insulin receptors (IRs) leading to a physiological (i.e. normal) activation of insulin receptor substrate 1 (IRS-1), and protein kinase B/Akt (Akt) and 44 and 42 kDa mitogen-activated protein kinases (p44/42^{mapk}). This phenomenon results in sustaining the activity of the endothelial nitric oxide synthase (eNOS) whose substrate L-arginine supply occurs via the activity of human cationic amino acid transporters (hCAT) (likely isoforms 1 and 2B). The role of Akt in the latter phenomenon is well documented, but p44/42^{mapk}-mediated activation of eNOS in HUVECs remains unclear (?). Activation of eNOS in response to insulin results in generation of NO leading to umbilical vein dilation (*Normal vasodilation*). In HUVECs from pregnancies where the mother showed with pre-gestational maternal obesity (*PGMO*), circulating insulin at the umbilical cord is higher leading to activation of IRs and subsequent inhibitory phosphorylation of IRS-1 resulting in reduced activity of this signalling protein. IRS-1 inactivation results in decreased activity of Akt ending in lower eNOS activity and NO generation. The consequence of the lower generation of NO leads to reduced umbilical vein dilation (*Reduced vasodilation*) in PGMO. The hCATs-mediated L-arginine transport is elevated in cells from PGMO, a phenomenon that is ineffective intending to counteract the reduced eNOS activity seen in this condition.

Francisco Mardones: I declare that I participated in making a critical reading of the manuscript. I have seen and approved the final version.

José A Poblete: I declare that I participated in making a critical reading of the manuscript. I have seen and approved the final version.

Luis Sobrevia: I declare that I participated in the development of the original idea, analysed the literature and results, drafted the manuscript, made a critical reading of the document, generate the first draft of the figures, and directly contribute to the discussion. I have seen and approved the final version.

Marcelo Farías: I declare that I participated in the development of the original idea, performed experiments, analysed the literature and results, drafted the manuscript, made a critical reading of the document, generate the first draft of the figures, and directly contribute to the discussion. I have seen and approved the final version.

Acknowledgements

Authors thank the midwives and personnel of the labour ward at the Hospital Clínico UC-CHRISTUS for the supply of placentas. This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico [grant numbers 1190316, 1150377, 1121145]. R Villalobos-Labra holds a PhD fellowship from Comisión Nacional para la Investigación en Ciencia y Tecnología (CONICYT) (Chile).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.07.007.

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