Modulation of placental protein expression of OLR1: implication in pregnancy-related disorders or pathologies

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Abstract

The lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (OLR1) is a newly described receptor for oxidatively modified LDL. The human pregnancy is associated with hyperlipidemia and oxidative stress. It has been reported that modification in maternal lipid profile can induce disturbance during pregnancy. In this study, we have evaluated the expression protein level of OLR1 in human term placenta of women having plasma cholesterol level lower to 7 mM or higher to 8 mM and women of gestational diabetes mellitus (GDM) by western blot analysis. The present study demonstrates that the maternal lipid profile is associated with placental protein expression of OLR1. A significant increase in the protein expression of OLR1 was observed in placenta of women with elevated plasmatic total cholesterol level (>8 mM). In addition, the placental protein expression of OLR1 is increased in mothers having the highest pre-pregnancy body mass index (BMI) and low (<7 mM) plasmatic total cholesterol level at term. Interestingly, the placental protein expression of OLR1 is increased in the presence of GDM pregnancies compared with normal lipids level pregnancies, without the modification of mRNA expression. In conclusion, placental OLR1 protein expression is associated with maternal lipid profile, pre-pregnancy BMI, and pathology of GDM.

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Introduction

The placenta is the central support organ for the developing fetus, serving as the site of maternal–fetal exchange of ions and lipids (Kanne *et al.* 2005). Human trophoblast differentiation is characterized by the formation of a specific multinuclear structure named the syncytiotrophoblast. The syncytiotrophoblast cells possess two structurally and functionally different plasma membranes: a brush border membrane (BBM) facing the maternal circulation and a basal plasma membrane (BPM) facing the fetal circulation (Lafond *et al.* 1988, Robidoux *et al.* 1998). During pregnancy, cholesterol is an essential component for placental and fetal development, used by the placenta for the synthesis of steroid hormones (Di Cianni *et al.* 2003). Physiological adaptations of maternal lipoprotein metabolism occur throughout pregnancy, leading to an increase in lipoprotein concentrations from second trimester to term. Maternal hypercholesterolemia is a characteristic feature during pregnancy and corresponds to an

accumulation of triglycerides (TGs) in very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL; Warth *et al.* 1975, Herrera 2002). Although the exact mechanism of cholesterol transport across the placenta remains unclear, many studies suggest the implication of HDL as well as LDL, both providing cholesterol for placental hormone synthesis (Gwynne & Mahaffee 1989). Early in pregnancy, maternal anabolic phase produces metabolic changes to support lipogenesis and fat storage in preparation for the catabolic phase of late pregnancy, a period of rapid fetal growth (Kaaaja 1998). The maternal insulin resistance observed during this period increases lipolysis in adipose tissue, leading to an enhanced flux of fatty acids to the liver. This promotes the synthesis of VLDL resulting in an increase in TG concentrations. Moreover, insulin resistance reduces the activity of lipoprotein lipase, the enzyme responsible for VLDL clearance from plasma. Then, VLDL remains in the plasma for longer period and ultimately leads to the

accumulation of LDL. Thus, this increase in LDL is directly associated with the development of atherosclerosis (Ross 1999).

Human placental cells express many lipoprotein receptors that can bind many lipoproteins such as the LDL receptor (LDLR; Cummings *et al.* 1982), the LDL receptor-related protein (LRP; Gafvels *et al.* 1992), the VLDL receptor (VLDL receptor; Wittmaack *et al.* 1995), the scavenger receptors (Bonet *et al.* 1995, Cao *et al.* 1997, Lafond *et al.* 1999), and the lectin-like oxidized LDL receptor-1 (OLR1; Yoshida *et al.* 1998). This latter one was recently cloned by Sawamura *et al.* (1997) and Moriwaki *et al.* (1998), and it can bind, internalize, and degrade the oxidized LDL (ox-LDL). OLR1 is a type II membrane glycoprotein belonging to C-type lectin family with a short N-terminal cytoplasmic tail and a long C-terminal extracellular domain (Kita *et al.* 2001). It has a molecular weight of 47 kDa and is initially synthesized as a 40 kDa precursor protein (pre-OLR1), that has a minimal N-linked high-mannose-type carbohydrate, which is further glycosylated into the mature protein and transported to the cell surface (Kataoka *et al.* 2000). OLR1 binds ox-LDL, delipidated, and solubilized ox-LDL, indicating that oxidatively modified apolipoprotein B-100 (apo-B) or some oxidized phospholipids firmly attached to the apo-B might be ligands of OLR1 (Moriwaki, *et al.* 1998). In addition, this receptor is upregulated by pro-atherogenic conditions, such as dyslipidemia, hypertension, and diabetes (Nagase *et al.* 1997, Kume *et al.* 1998, Chen *et al.* 2000a, 2000b, 2001).

During human pregnancy, the maternal lipoprotein profiles are greatly modified (Herrera 2002). Fundamentally, pregnancy is associated with an oxidative stress state arising from the increasing metabolic activity of placental mitochondria and the decreasing scavenging by antioxidants (Wisdom *et al.* 1991). Products of lipid peroxidation have been detected in human placenta; LDLs are one of the main targets for lipid peroxidation and are converted to their oxidized form (Fialova *et al.* 2002). This process represents a key step in the development of atherosclerosis, since several studies showed that LDLs become atherogenic when they are converted to ox-LDL (Steinberg 1997, Witztum & Horkko 1997). Because they have reduced affinity for the native LDLR, smaller and denser particles are considered to be more atherogenic. Thus, they are longer retained in the circulation, and become more inclined to oxidation, maybe because they contain less intrinsic antioxidants (Dejager *et al.* 1993, Rajman *et al.* 1999). ox-LDL becomes a ligand for scavenger receptors, including OLR1.

Affecting 3–7% of pregnant women, gestational diabetes mellitus (GDM) is characterized by endothelial dysfunction and reactive nitrogen and oxygen species, which contribute to the progression of diabetes (Honing *et al.* 1998, Rosen *et al.* 2001). In diabetes, reactive oxygen species (ROS), including superoxide, are thought to be

produced as a result of prolonged periods of exposure to hyperglycemia, known to cause non-enzymatic glycation of plasma proteins (Tames *et al.* 1992). In addition, GDM is generally associated with disturbances in lipoprotein metabolism with a tendency toward hypercholesterolemia (Taskinen 1990). During pregnancy, women with GDM have higher serum TG concentrations, but lower HDL cholesterol concentrations than normal pregnant women (Koukkou *et al.* 1996).

We hypothesize that maternal lipid profile could modify placental expression of OLR1. Therefore, this study was designed to characterize the metabolic modifications of circulating lipids on the expression and localization of OLR1 in human term placenta of normal, hypercholesterolemic, and GDM pregnancies, and potentially correlate the modifications with BMI and some inflammatory cytokines. Thus, this study will provide better understanding of the regulation of important receptor involved in oxidized cholesterol metabolism and pregnancy-related disorders/pathologies, in which the lipid profile may be disturbed.

Results

Subject characteristics

Basic characteristics of mothers and newborns are given in Table 1. A total of 72 women gave birth and their blood samples were collected at term. The participants were split into two groups: low cholesterol (LC) women ($n=43$) and high cholesterol (HC) women ($n=29$). These groups were very similar according to the age of the women (± 32 years old), their gestational age at delivery (± 39 weeks), and the mothers' BMI (± 24 kg/m²). Also, no differences between groups were observed concerning the mothers' weight gain, the babies' weight and height at birth, and the placental weight.

Plasma lipids at birth and in cord blood

Table 2 shows total cholesterol, HDL cholesterol, LDL cholesterol, TG, apo-AI, and apo-B concentrations at delivery and in cord blood for LC and HC groups. Total cholesterol ($P<0.001$), LDL cholesterol ($P<0.001$), TG ($P=0.0029$), and apo-B100 ($P<0.001$) concentrations

Table 1 Population characteristics.

	LC group (<7 mM) $n=43$	HC group (>8 mM) $n=29$
Mother age (year)	31.2 ± 5.3	31.2 ± 4.2
Gestational age (week)	39.0 ± 1.5	39.3 ± 1.5
Mother BMI (kg/m ²)	23.5 ± 5.0	22.41 ± 4.36
Mother weight gain (kg)	15.01 ± 6.09	18.83 ± 6.81
Newborn birth weight (g)	3291 ± 648	3420 ± 462
Newborn height (cm)	51.5 ± 2	52.0 ± 5.0
Placenta weight (g)	592.4 ± 154.1	609.8 ± 142.7

Results are expressed as mean $\pm$ s.d.

Table 2 Plasma lipids at delivery and in cord blood.

	LC group (<7 mM) n=43		HC group (>8 mM) n=29	
	At delivery	Cord blood	At delivery	Cord blood
Total cholesterol (mM)	5.75±1.6	1.88±0.94	8.21±1.00 [†]	1.68±0.51
HDL cholesterol (mM)	1.70±0.46	0.75±0.31	1.76±0.57	0.73±0.31
LDL cholesterol (mM)	2.80±0.84	0.84±0.54	4.84±0.94 [†]	0.67±0.28
TG (mM)	2.71±1.12	0.64±0.52	3.59±1.21*	0.60±0.27
Apo-AI (g/l)	2.08±0.38	0.90±0.36	2.21±0.46	0.83±0.23
Apo-B100 (g/l)	1.14±0.26	0.30±0.23	1.64±0.25 [†]	0.26±0.10

Results are expressed as mean±s.d., where * $P<0.01$, [†] $P<0.001$ compared with the maternal LC group.

were significantly increased in HC women at delivery. The concentrations of HDL and apo-AI showed no differences between both groups. Also, no differences were observed in cord blood sample for both groups regarding all studied parameters.

Protein expression of OLR1

The expression of OLR1 was evaluated by western blot using total protein isolated from placental tissue. Figure 1A shows a representative western blot of the expression of OLR1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Figure 1 Representative protein expression of OLR1 and inflammatory cytokines concentration from total placental proteins extracts according to maternal plasmatic total cholesterol concentration. (A) Western blot analysis was performed using OLR1 antibody on total protein (150 µg) isolated from placental tissue. Thereafter, the blot was stripped and reprobed with an anti-GAPDH antibody. (B) Densitometric analysis of OLR1 protein level after normalization with GAPDH protein level for LC ($n=43$) and HC ($n=29$) groups. (C) Inflammatory cytokines IL1B (right) and TNF (left) content in term placenta for LC ($n=31$) and HC ($n=21$) groups. Results are expressed as mean±s.e.m., where *** $P<0.001$.

from placental tissue extracts. Following normalization with the GAPDH protein level, our results demonstrate that the expression of OLR1 is significantly increased (68%) in HC group compared with LC one (Fig. 1B). Figure 2 shows the expression of OLR1 after its normalization with GAPDH, in relation to different (A) pre-pregnancy BMI (<20 kg/m², 20–26 kg/m² and >26 kg/m²). For the LC groups (white bars), a significant increase in OLR1 protein expression in placenta of women with the highest BMI is observed compared with those with the lowest BMI ($P=0.0072$), while for the HC women (black bars), no differences were observed between groups. In addition, our results show an increase in the protein expression of OLR1 between the LC and HC groups for the two lowest BMI groups ($P=0.0002$ and $P<0.0001$, respectively).

Level of inflammatory cytokines in term placenta

To evaluate the role of inflammatory markers on OLR1 placental protein expression, the quantification of interleukin 1β (IL1B) and tumor necrosis factor-α (TNF) was done. These cytokines are highly expressed in placenta, principally IL1B. Figure 1C shows the IL1B and TNF concentrations in human term placenta in relation to maternal plasmatic total cholesterol level at term. Our results demonstrate that these cytokines were not influenced by maternal plasmatic total cholesterol level, were neither influenced nor by maternal pre-pregnancy BMI (Fig. 2B). Finally, in HC women, placental IL1B level was influenced by weight gain during pregnancy (data not shown). Specifically, an increase of ±65% is observed between women who gained >18 kg compared with women with normal weight gain (11–18 kg; 235.2 ± 38.03 vs 139.2 ± 11.54 respectively). No difference was showed for TNF related to weight gain (data not shown).

Protein expression of OLR1 in syncytiotrophoblast membranes

The expression of OLR1 was evaluated by western blot in syncytiotrophoblast plasma membranes (BPM and BBM), isolated from fresh placenta. Figure 3 shows representative western blot of the expression of OLR1 in BPM, BBM, syncytiotrophoblast cells, and total protein from placental tissue. Expression of OLR1 protein, which

Figure 2 Representative protein expression of OLR1 and inflammatory cytokines concentration from total placental proteins extracts according to maternal pre-pregnancy BMI. (A) Densitometric analysis of OLR1 protein level after normalization with GAPDH protein level. Western blot analysis was performed using OLR1 or GAPDH antibody in total proteins (150 µg) extracted from placental tissue. (B) Inflammatory cytokines IL1B (top) and TNF (bottom) contents in term placenta. Results are expressed as mean $\pm$ S.E.M., where $^{++}P < 0.01$ and $^{***}P < 0.001$.

has a molecular weight of 47 kDa, was detected in both placental membranes, in syncytiotrophoblastic cells and in total protein extract from placenta.

Immunohistochemical study of OLR1

As shown in Fig. 4, immunohistochemical study of OLR1 expression in human placenta demonstrated that it was localized in both maternal BBM and fetal BPM sides of

Figure 3 Representative protein expression of OLR1 in human placental tissues. Proteins (150 µg) were migrated on an 8% SDS-PAGE under reducing conditions. Blots were done with OLR1 antibody. Maternal side purified membranes (BBM), fetal side purified membranes (BPM), syncytiotrophoblast (STB; $n=3$), and total protein extract from placental tissue (PLA; $n=3$).

syncytiotrophoblast cells and in fetal capillaries (Fig. 4A). OLR1 stained strongly immunopositive principally in BBM.

Correlations between OLR1 protein expression and circulating plasma lipids

The correlation between OLR1 protein expression and many maternal plasma circulating lipids was evaluated by Spearman's correlation and Pearson's linear correlation curves. Positive correlations were observed between OLR1 protein expression and maternal plasmatic total cholesterol level ($r=0.4248$, $P=0.001$), LDL plasma level ($r=0.4102$, $P=0.0019$), and apo-B100 level ($r=0.3885$, $P=0.0018$) at term, as shown in Fig. 5A, B and D (left plots) respectively. By contrast, no correlation was marked between the OLR1 protein expression and maternal plasma HDL level ($r=0.1292$, $P=0.4619$) at term (Fig. 5C). In addition, no correlation was found between OLR1 protein expression and cord blood total plasma cholesterol ($r=-0.0773$, $P=0.5725$), LDL cholesterol ($r=-0.1680$, $P=0.3737$), HDL cholesterol ($r=-0.03027$, $P=0.7236$) and apo-B100 ($r=0.07784$, $P=0.8223$) levels (Fig. 5A–D, right plots).

Population characteristics for women with GDM

Basic characteristics for mothers and newborns are given in Table 3. A total of 24 women gave birth and their blood samples were collected at term. The participants were split into two groups: control women ($n=15$) and GDM women ($n=9$). The age of the women was ± 31 years old, and their gestational age was about 39 weeks. Compared with controls women, the GDM women had significant highest BMI (23.2 ± 0.7 vs 27.8 ± 1.9 ; $P=0.0242$). No differences were observed according to the maternal weight gain, weight and height at birth for babies of both groups, and placental weight.

Plasma lipids during pregnancy and at birth of GDM women

Table 4 presents total cholesterol, HDL cholesterol, LDL cholesterol, TG, apo-AI, and apo-B100 concentrations at

Figure 4 Immunohistochemical expression of OLR1 in human placental tissue. Shortly, frozen placental tissues were cut and fixed on glass slides. After being incubated with primary antibodies, slides were stained and viewed under interference contrast microscope. Positive staining is in brown color and nuclear counterstaining is blue. (A) OLR1 is present in both maternal (BBM) and fetal (BPM) sides of syncytiotrophoblast (STB) from term placenta as well as in fetal capillary (FC). (B) The positive control (hCG) is present in syncytiotrophoblast while (C) the endothelial marker, CD34, is present on fetal capillaries (FC). (D) Negative control (NC) consists of placental tissue without primary antibody.

delivery and in cord blood for control and GDM women. No differences were observed for total cholesterol, LDL cholesterol, HDL cholesterol, apo-AI, and apo-B100 at delivery. However, GDM subjects had significantly higher concentration of TG at delivery compared with control women (2.26 vs 3.17; $P=0.0077$). Also, no differences were observed in cord blood samples for total cholesterol, HDL cholesterol, TG, apo-AI, and apo-B100, while GDM condition induced a significant decrease in total cholesterol (1.99 vs 1.65; $P=0.0407$) and LDL cholesterol (1.03 vs 0.68; $P=0.0068$) in cord blood.

mRNA expression of OLR1 in GDM condition

The expression of *OLR1* was evaluated by real-time PCR using mRNA isolated from placental tissue. Figure 6 presents the relative quantification, using 18S as reference gene. We observed no difference in GDM women compared with control ones (0.6422 vs 0.6591).

Protein expression of OLR1 in GDM women

The expression of OLR1 was evaluated by western blot using total protein isolated from placental tissue. Figure 7A shows representative western blot of the expression of OLR1 and GAPDH from placental tissue ($n=3$ for each group). Following normalization with GAPDH protein level, our results (Fig. 7B) show that the expression of OLR1 in GDM women is significantly increased by 53% compared with the control ones (0.5354 vs 1.006; $P=0.0015$).

Inflammatory cytokines level in term placenta of women with GDM

Figure 7C shows the concentration of IL1B and TNF in term placenta of GDM and CTL women. Our results clearly demonstrated that these cytokines were modified in GDM group since both placental IL1B and TNF were significantly increased (0.0139 and 0.0437 respectively).

Discussion

Our study shows that maternal lipid profile is associated with OLR1 protein expression in human term placenta. Specifically, placental OLR1 protein expression is increased in case of maternal hypercholesterolemia and GDM, and is affected by pre-pregnancy BMI. In addition, in the present study, we show for the first time the expression of OLR1 in both maternal BBM and fetal sides BPM of normal human placenta.

Abnormally high plasma lipid concentration is thought to be an important pathogenic factor, principally in atherogenesis. It is known that OLR1 expression is increased in case of hypertension, atherosclerosis, and hyperlipidemia *in vivo* (Mehta *et al.* 2006). A study using apo-E knockout mice, a model for spontaneous hypercholesterolemia, the OLR1 expression is reduced by feeding mice with a HMG-CoA reductase inhibitor (Chen *et al.* 2006), which decreases LDL cholesterol level and reduces the formation and uptake of ox-LDL (Chen *et al.* 2000a, 2000b). Our data are in accordance with these observations since we showed a positive correlation between plasma total cholesterol, LDL cholesterol, and apo-B100 levels with placental protein

Figure 5 Relationship between OLR1 protein expression and maternal plasmatic lipids concentration at delivery and in cord blood. (A) Total cholesterol ($n=83$), (B) LDL cholesterol ($n=68$), (C) HDL cholesterol ($n=70$), and (D) apo-B100 ($n=79$). Results are expressed as Spearman's correlation and the curve represent Pearson's linear correlation.

expression of OLR1. In contrast to non-pregnant women, there is a physiological change in plasma lipids and an increased oxidative turnover that is controlled by countervailing anti-oxidative mechanisms in healthy pregnant women (Toescu *et al.* 2002). In response to the high demand by the fetus, there is an increase production of ROS by the placenta during pregnancy. On the other hand, placenta is a source of an anti-oxidative enzyme and hormone system that controls placental lipid peroxidation in healthy pregnancy (Gitto *et al.* 2002). Many studies reported higher levels of placental lipid peroxides in pre-eclampsia than in normal pregnancies (Walsh & Wang 1993, Gratacos *et al.* 1998, Serdar *et al.* 2002), suggesting that placental tissues could be the source of elevated levels of serum

Table 3 Population characteristics of women with gestational diabetes mellitus.

	Control group ($n=15$)	GDM group ($n=9$)
Mother age (year)	32.5 ± 3.6	30.9 ± 1.6
Gestational age (week)	40.1 ± 0.4	39.2 ± 0.2
Mother BMI (kg/m^2)	23.2 ± 0.7	$27.8 \pm 1.9^*$
Mother weight gain (kg)	13.5 ± 1.0	11.8 ± 1.6
Newborn birth weight (g)	3591 ± 121.5	3607 ± 99.8
Newborn height (cm)	52.0 ± 0.6	51.8 ± 0.5
Placenta weight (g)	554.5 ± 34.7	598.1 ± 71.7

Results are expressed as mean $\pm$ s.d. where $^*P < 0.05$ compared with control group.

lipid peroxides. Finally, it has been reported that OLR1 was the principal scavenger receptor responsible for the uptake of ox-LDL in placental cells (Pavan *et al.* 2004). Thus, the high level of OLR1 placental protein expression can be explained by the enhancement of oxidative stress in HC women. Obviously, the importance of oxidative stress in pregnant women and the lack of knowledge in this population justify pursuing studies in this particular field.

In this study, we demonstrated that pre-pregnancy BMI significantly increased placental OLR1 protein expression in LC women. We first hypothesized that this increase could be attributed to the inflammatory status observed in obesity (Chen 2006). It is well known that adipose tissue is a highly active endocrine organ secreting a number of hormones that alter the circulation of metabolites, cytokines, and growth factors (Grundy 2004, Havel 2004). Thus, obese women are more likely than normal weight women to enter pregnancy in a subclinical inflammatory state since increases in body fat are associated with elevated cytokine levels and inflammation (Greenberg & Obin 2006). Surprisingly, our data showed no difference for both IL1B and TNF in relation to maternal BMI. However, this effect could be attributed to the fact that maternal adiposity could also produced a hypoxic state when glycosylated hemoglobin levels are increased and affinity for oxygen is reduced, decreasing oxygen transfer to the uterus and impairing placentation (King 2006). Thus, many molecules related to oxidative stress can induce the expression of OLR1, such as isoprostanes, which are prostaglandin isomers formed by free radical peroxidation of arachidonic acid presents in phospholipids (Sakurai & Sawamura 2003). Among these isoprostanes, 8-iso-prostaglandin $F_2\alpha$, a well-known marker of oxidative stress (Sakurai & Sawamura 2003), has the potential to increase the uptake of oxidized LDL and the expression of OLR1 in human placenta choriocarcinoma JAR cells (Halvorsen *et al.* 2001). Some of our data showed that maternal weight gain had no effect on placental OLR1 protein expression. In contrast, our results demonstrated an increase in IL1B content for HC women with normal weight gain (11–18 kg) compared

Table 4 Plasma lipids during pregnancy and in cord blood of women with gestational diabetes mellitus.

	Control group (n=15)		GDM group (n=9)	
	At delivery	Cord blood	At delivery	Cord blood
Total cholesterol (mM)	6.41 ± 0.23	1.99 ± 0.1	6.22 ± 0.32	1.65 ± 0.10*
HDL cholesterol (mM)	1.85 ± 0.11	0.72 ± 0.06	1.62 ± 0.11	0.69 ± 0.04
LDL cholesterol (mM)	3.52 ± 0.19	1.03 ± 0.08	3.15 ± 0.20	0.68 ± 0.06†
TG (mM)	2.26 ± 0.14	0.51 ± 0.04	3.17 ± 0.32†	0.61 ± 0.12
Apo-AI (g/l)	2.09 ± 0.08	0.89 ± 0.04	2.10 ± 0.09	0.82 ± 0.05
Apo-B100 (g/l)	1.23 ± 0.04	0.29 ± 0.04	1.26 ± 0.07	0.22 ± 0.03

Results are expressed as mean ± s.d., where * $P < 0.05$, † $P < 0.01$, compared with control group.

with HC women with lowest weight gain (<11 kg). The precise relationship between this cytokines and maternal weight gain remains unclear and further investigations will be necessary.

The present study demonstrates the localization of OLR1 in both maternal BBM and fetal BPM sides of syncytiotrophoblasts. The binding of ox-LDL with OLR1 induces superoxide generation, inhibits NO production, enhances endothelial adhesiveness for leucocytes, and induces chemokine expression (Cominacini *et al.* 2000, Li & Mehta 2000). Thus, the presence of OLR1 in both sides of syncytiotrophoblasts may promote apoptosis (Lee *et al.* 2005). A recent study shows that the functional role of trophoblasts in placental dysfunction results from the localization and the upregulation of OLR1 in pre-eclamptic placenta, which possibly promotes apoptotic activity (Lee *et al.* 2005). Since OLR1 is a receptor for ox-LDL, a highly atherogenic lipoprotein, physiological functions of placental cells may be altered. Thus, maternal hypercholesterolemia could promote fetal atherosclerosis development via OLR1. Finally, further investigations will be required to understand the precise role of these two binding sites.

GDM induces a state of dyslipidemia consistent with insulin resistance. Indeed, during pregnancy, GDM women had higher plasma TG concentrations (Butte 2000). In normal pregnancy, the higher concentration of estrogens and insulin resistance are considered to be responsible for the hypertriglyceridemia (Butte 2000),

while in GDM women, increased insulin resistance may account to the rise in TG concentration (Koukkou *et al.* 1996). In this study, we found an increase in TG concentration at delivery and a decrease in total and LDL cholesterol in cord blood of GDM women. A novel study shows that GDM increased the LDL susceptibility to oxidation, a susceptibility due to diabetes *per se*, as well as the increase in incidence of obesity found in these women (Sanchez-Vera *et al.* 2007). This fact is in accordance with our results, since we found that GDM women had higher BMI than control ones. It is known that hyperlipidemia is exaggerated in obese pregnant women (Ramsay *et al.* 2002). This fact could be attributed to the inability of insulin to suppress whole-body lipolysis, leading to a marked increase in plasma

Figure 6 Quantification of OLR1 mRNA level in human term placenta. Quantification was performed by real-time PCR for CTL (n=15) and GDM (n=9). Briefly, total RNA was extracted from placental tissue and RT was performed in a 20 µl reaction containing 0.5 µg total RNA and the Oligo-dT primers. The PCRs were performed using 0.5 µM of both sense and antisense primers, for 55 cycles. Results are expressed as mean ± S.E.M.

Figure 7 Representative protein expression of OLR1 and inflammatory cytokines content extracts from human term placenta. (A) Western blot analysis of OLR1 on total protein (150 µg) isolated from placental tissue. Thereafter, the blot was stripped and reprobed with an anti-GAPDH antibody. (B) Densitometric analysis of OLR1 protein level in CTL (n=10) and GDM (n=6) women after normalization with GAPDH protein level. (C) Inflammatory cytokines, IL1B (right) and TNF (left), content in term placenta of CTL (n=10) and GDM (n=6) women. Results expressed as mean ± S.E.M., where ** $P < 0.01$ and * $P < 0.05$.

free fatty acids in obese women compared with control women (Xiang *et al.* 1999). The decrease in total cholesterol and LDL cholesterol concentrations, found in cord blood of babies from GDM women, may be attributed to a decrease in the placental blood flow and placental damage due to lipid peroxides generated by the increased LDL oxidation. As a result, this would compromise the supply of nutriment toward the fetus. An important finding of our study is that GDM increases placental OLR1 protein expression without affecting mRNA expression levels. This result, for the protein expression, is in accordance with other studies that demonstrated the increase in OLR1 expression in different tissues of diabetic subjects. It has been reported that OLR1 expression is increased by glucose both in macrophage and endothelial cells (Li *et al.* 2003, 2004). Effectively, diabetes is characterized by the presence of an important oxidative stress, endothelial dysfunction, and increased expression of endothelial molecules in the inflammatory cells (Stevens 2005). Thus, this effect could be attributed to the maternal inflammatory status; in a recent study, an increase in cytokines concentration in maternal blood with GDM was observed (Heitritter *et al.* 2005). This finding agrees with our results where we demonstrated a significant increase in two central mediators of inflammatory reaction, IL1B and TNF, in placenta of women with GDM. Finally, we show that this increase is more pronounced for placental IL1B, while this cytokine is associated with the risk of developing type 2 diabetes (Spranger *et al.* 2003). In fact, several studies show that proinflammatory cytokines regulate the expression of OLR1 (Kume *et al.* 2000, Chiba *et al.* 2001, Hofnagel *et al.* 2006). A study using smooth muscle cells showed that strong co-distribution of proinflammatory cytokines, IL1A and IL1B, and TNF with OLR1 expression promotes atherosclerosis lesions (Hofnagel *et al.* 2006). Moreover, pregnancy induces inflammatory changes characterized by the activation of peripheral blood leucocytes (Sacks *et al.* 1998), and the production of pro-anti-inflammatory cytokines such as IL6 and TNF. Finally, this increase in OLR1 expression observed in women with GDM could be regulated by a post-transcriptional mechanism implying these cytokines. Finally, babies delivered under GDM were normal, resulting in the maternal intake of insulin during the third trimester of pregnancy (Challier *et al.* 1986). To conclude, all of these facts are in accordance with our data, where we found that placental protein expression of OLR1 is increased in women with GDM.

In summary, our results corroborate the importance of the maternal lipid profile during pregnancy. Thus, maternal hypercholesterolemia, as well as GDM, could promote fetal atherosclerosis development via an upregulation of placental expression of OLR1. In addition, we reported for the first time the presence of OLR1 in both maternal and fetal sides of syncytiotrophoblasts. Taking together, these data suggest that the post-transcriptional

modulation of placental protein expression OLR1 by maternal lipid profile disturbance or pathologies during pregnancy can ultimately influence fetal growth and development.

Materials and Methods

Study population

The women participating in the study were recruited at their first prenatal visit, before their tenth week of pregnancy, at the Clinique Fidès of Montreal and at the Service of Perinatology, of the Centre Hospitalier de l'Université de Montréal (CHUM), Pavilion St-Luc, QC, Canada, from 2002 to 2006. After signing a consent form, each woman filled out an interview-administrated questionnaire, which contained general socio-demographic data, medical history, drinking, and smoking habit. The total study population consisted of 900 pregnant women. To conduct this study, 81 women were selected. Of these women, 72 had a normal pregnancy, while 9 developed GDM. The ones from normal pregnancy were classified into two groups according to the plasmatic total cholesterol concentration at term. To establish the groups, we established the median of maternal plasmatic total cholesterol at term (6.42 mM). Women with lower than 7 mM referred to the LC group ($n=43$) and women with cholesterol concentration higher than 8 mM referred to the HC group. Subsequently, they were reclassified into three groups according to their pre-pregnancy body mass index (BMI). The BMI, representing a measure of adiposity, was calculated as pre-pregnancy kg/m^2 . The established normal values for BMI, according to the Health Canada (2002), were between 20 and 26 kg/m^2 . Women who developed GDM ($n=9$) were matched with 15 control (CTL) women giving birth between 37 and 40 weeks of pregnancy, having normal lipid profile, normal values for BMI, and weight gain. Screening for diabetes pregnancy was done according to the CHUQ and CHUM protocols (Forest *et al.* 1983, 1994). Pregnant women were screened for GDM between 24 and 32 weeks with the 50 g glucose challenge test. The subject having a serum glucose values equal or greater than 7.8 mmol/l, women were tested with the 100 g oral glucose tolerance test. All of these women were non-smokers and did not receive any medication known to interfere with lipid metabolism. Finally, a post-natal follow-up was made to assess newborns' health status as well as collecting data relative to the weight and height of the babies.

Blood samples

Blood samples were collected at delivery, from the mother and the cord blood. The blood samples were collected in 10 ml gel Vacutainer tube gel (BD, Oakville, Canada) and centrifuged for 15 min at 3500 g , less than 1 h after delivery. Plasma samples were kept at -20°C until analysis. The placentas, from vaginal delivery, were obtained from the collaborating hospital and immediately immersed in Dulbecco's modified Eagle's medium (Sigma) containing a mixture of antibiotic (penicillin, streptomycin, and neomycin; Invitrogen) and NaHCO_3 . After the removal of the amnion, chorion and decidual layers, the

placental tissue was cut into sections of 5 cm² and immediately frozen in liquid nitrogen and kept at -80°C until use.

Lipid assays

The plasma levels of total cholesterol and LDL, HDL, VLDL, and TG were individually measured using the Unicel 36 DX600 Synchron Clinical System (Beckman-Coulter, Mississauga, ON, Canada), at the Clinical Biochemistry Service of Hôpital Saint-François d'Assise, Québec (QC, Canada).

Total proteins extraction

The placental frozen tissue samples were washed three to four times with 0.9% cold NaCl solution containing anti-protease (1 μM leupeptin, 1.46 μM pepstatin, and 2 $\mu\text{g}/\text{ml}$ aprotinin) to remove blood from tissue. The tissue (1 g) was homogenized using a Polytron tissue homogeniser PT 3000 (Brinkmann, Canada) in 1 ml ice-cold hypertonic buffer (125 mM Tris-HCl, pH 8.0, 2 mM CaCl_2 , 1.4% (v/v) Triton X-100, 1 μM leupeptin, 1.46 μM pepstatin, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM phenylmethylsulphonylfluoride (PMSF)). The homogenate was kept on ice for 30 min, centrifuged at 10 000 g for 25 min at 4°C , the supernatant was collected, and stored at -80°C until used. The protein concentration content was determined by spectrophotometric quantification using the bicinchoninic acid reagent (Pierce, Brockville, ON, Canada) with BSA as standard.

Purification of syncytiotrophoblast BBM and BPM

Briefly, membranes were purified from placental tissues collected from fresh human placentas obtained from full-term normal vaginal delivery (CHUM, Pavilion St-Luc, QC, Canada). After the removal of the amnion, chorion and decidual layers, the tissues were minced and stirred for 45 min in 10 mM Tris-HEPES (pH 7.4) containing 270 mM mannitol, 0.1 mM PMSF, 1 mg/ml benzamidin, and 10 mM leupeptin. The membranes were purified, as described by our laboratory (Lafond *et al.* 1988) with some modifications (Robidoux *et al.* 1998).

Western blot analyses of OLR1

Total placental proteins (150 μg for total placental protein and 30 μg for syncytiotrophoblastic membranes) were solubilized in a sample buffer (4% SDS, 30 mM dithiothreitol, 10% β -mercaptoethanol, 0.25 M sucrose, 0.01 M EDTA- Na_2 , and 0.075% bromophenol blue) and heated at 95°C for 5 min. The proteins were resolved in a 8% SDS-PAGE and electroblotted to PDVF membrane (Millipore, Cambridge, ON, Canada) at 20 V for 40 min. Membranes were blocked overnight at 4°C in TBS-T (20 mM Tris base, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% skimmed milk. Thereafter, the membranes were washed three times with TBS-T and incubated with anti-human OLR1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1/1000 in TBS-T/5% BSA and 1% skimmed milk), or human GAPDH mouse MAB (Chemicon International, Temecula, CA, USA; 1/5000 in TBS-T/5% BSA) for 90 min at room temperature. Blots were washed three times with TBS-T,

and probed with horseradish peroxidase-conjugated secondary antibodies, for OLR1, anti-goat IgG (1/6500 TBS-T 5% BSA and 1% skimmed milk) and for GAPDH anti-mouse IgG (1/3000 TBS-T 5% skimmed milk; Chemicon International) for 90 min at room temperature. Blots were then washed three times with TBS-T and the detection was performed using the BM chemiluminescence system (Roche Diagnostics) and visualized by autoradiography (Hyperfilm ECL, GE Healthcare, Baie d'Urfée, QC, Canada). The PDVF membranes were stripped with a stripping solution (12 M HCl (pH 2.0) 25 mM glycine, and 1% SDS) for 10 min at room temperature. Thereafter, the membranes were rinsed three times with a washing solution (1 M Na_2HPO_4 , 1 M NaH_2PO_4 (pH 7.2), and 9 g/l NaCl) for 5 min and blocked for 1 h. The membranes were then ready to be reprobed with GAPDH for loading standardization. We normalized data with a control sample that served to express data as percentage of control. For semi-quantitative analyses of the bands, the film was digitized and analyzed by the Quantity One Software (Bio-Rad Laboratories).

RNA analysis

Total RNA was extracted from placental tissue using the High Pure RNA Tissue Kit (Roche Diagnostics). The RT was performed in a 20 μl reaction containing 0.5 μg total RNA using the Omniscript kit (Qiagen) and the Oligo-dT primers (Roche Diagnostics) according to the manufacturer's instructions. The real-time PCRs were realized using the 480 SYBR Green I Master (Roche Diagnostics) on a LightCycler 480 Instrument (Roche Diagnostics). The PCRs were performed using 0.5 μM of both sense and antisense primers, for 55 cycles to ensure exponential amplification phase of the targeted genes. Primer sequences designed for human OLR1 were: (5'-3') (sense) TTACTCTCCATGGTGGTGGTGCC and (antisense) AGCTTCTTCTGCTTGTGTC. Human 18S primers, as reference gene, were: (5'-3') (sense) AGAGCTAATACATGCCGAC and (antisense) AGTTGATAGGCGACACG.

Immunohistochemistry

An immunohistochemistry study was performed to investigate the localization of OLR1 protein. We used appropriate placental tissues from fresh placentas. After the removal of the amnion, chorion and decidual layers, the placental tissue was cut into sections of $5 \times 5 \times 5$ mm, embedded in cold (4°C) Tissue-tek OCT (Electron Microscopy Sciences, Hatfield, PA, USA) and immediately frozen in isopentane at -80°C (Sigma-Aldrich). The sections were stored at -80°C until further processing. For immunohistochemical staining, frozen sections (8 μm thick) were mounted onto SuperFrost Plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and air-dried overnight. The sections were then fixed with freshly prepared cold 4% paraformaldehyde and 0.2% picric acid in PBS for 20 min at 4°C . To complete fixation, cryosections were air-dried at room temperature. Tissue sections were rinsed with PBS for 3 min and the sections were incubated with permeabilization solution (1% Triton X-100 in PBS) for 30 min at room temperature. The sections were blocked in 0.5% donkey serum and 0.1% Tween-20 for 60 min at room temperature, after quenching endogenous peroxidase activity by exposing

slides to 'Peroxidase Block' (Kit LSAB+ System-HRP; DAKO, Mississauga, ON, Canada) for 5 min. The sections were then incubated with primary antibodies diluted in blocking solution (Biomed, Foster City, CA, USA) overnight at 4 °C. The primary antibodies used were goat polyclonal anti-OLR1 antiserum (1:100, Santa Cruz Biotechnology), rabbit polyclonal anti-hCG (1:500, Chemicon), and mouse anti-CD34 (1:1000, NeoMarker, Fremont, CA, USA). To ascertain protein-specific antibody binding, controls (CTL) were performed omitting primary antibodies. Subsequently, they were incubated with endogenous avidin and biotin blocking solutions for 15 min at room temperature each. Tissue sections were incubated with 'Link' universal secondary antibody solution (DAKO) for 60 min at room temperature and then with 'streptavidin-peroxidase' for 30 min. They were stained with 'substrate-chromogen solution' (DAKO), and counterstained with Mayer's hematoxylin (Fluka, Buchs, Switzerland). The sections were viewed and photographed under interference contrast Leitz Diaplan microscope equipped with a Nikon CoolPix 990 camera. Cells having intense brownish staining were considered immunopositive.

Cytokine quantification

Interleukin-1 β (IL1 β) and TNF concentrations were measured in the total protein fraction extracted from placental tissue, using the Human IL-1 (IL-1 β) ELISA Ready-SET-Go! and Human TNF α (TNF) ELISA Ready-SET-Go! Kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analyses

Data were expressed as the mean $\pm$ S.E.M., and analyzed with unpaired Student's *t*-test at $P < 0.05$ level of significance, to evaluate difference between groups. For the relationship between two variables of the same population, the results are expressed as Spearman's correlation and the curve represent Pearson's linear correlation. All statistical analyses were performed using the Prism software (version 4.0.2, 2004; GraphPad Software, La Jolla, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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