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Oxidative stress as common trait of endothelial dysfunction in chorionic arteries from fetuses with IUGR and LGA

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ABSTRACT

Introduction: Fetal macrosomia and intrauterine growth restriction (IUGR) associate with increased morbidity in the neonate. Placental vascular relaxation is impaired in fetal macrosomia, as well as in IUGR, and this could result from increased oxidative stress present in both conditions. We determined the role of pro- and anti-oxidants on NOS dependent relaxation in placental chorionic arteries from pregnancies with LGA babies from overweight and/or obese mothers (LOOM) and IUGR fetuses from normal BMI women.

Methods: Chorionic arteries were mounted in a wire-myograph, where responses to the NOS-dependent agent CGRP in presence or absence of the antioxidant N-acetyl cysteine (NAC), the pro-oxidant SIN-1, the SOD inhibitor DDC, and the GPx inhibitor MS were determined. Additionally the presence of pro- and antioxidant enzymes (NOX-4, SOD-1, SOD-2 and GPx-1) and eNOS in chorionic and umbilical vessels were addressed by immunohistochemistry.

Results: Maximal CGRP-induced relaxation was comparable to controls but presented a reduced potency in chorionic arteries from LOOM placentae, whilst in IUGR vessels both maximal response and potency were reduced. NAC increased maximal relaxation in controls, IUGR and LOOM arteries, whilst SIN-1 completely abolished the CGRP-induced relaxation only in IUGR and LOOM samples, the later effect was paralleled by SOD or GPx inhibition. These responses associated with the presence of NOX-4, SOD-1 and GPx-1 in the endothelium and vascular wall of chorionic and umbilical arteries in the different groups studied.

Discussion: These data suggest that NOS dependent relaxation in placental vessels from IUGR and LOOM pregnancies present a higher sensitivity to oxidative stress.

1. Introduction

Both extremes of fetal growth trajectories, fetal macrosomia (>4000 g) or large for gestational age (LGA) newborns resulting from mothers with overweight/obesity (MO), and intrauterine growth restriction (IUGR) are associated with increased risk of morbidity in the neonate [1], and the development of cardiovascular diseases in the adulthood [2-4]. Compelling evidence shows that in IUGR pregnancies placental vascular function is impaired, however the effect of maternal overweight and obesity has not been described so far [1]. However current data suggests that increased maternal body mass index (BMI) since early stages of gestation associates with altered proteome profile [5], increased markers of inflammation [6] and oxidative stress [7,8] in the placenta. A recent study showed that chorionic plate arteries from placentae of obese women present a reduced relaxation in response to exogenous nitric oxide (NO) compared with placentae from women with normal BMI [9], suggesting an altered vascular function in the placenta of women with high BMI during pregnancy. Nonetheless there is no data showing whether NO synthase- (NOS) dependent relaxation is impaired in placentae from LGA fetuses and which mechanisms would be participating in this effect.

Abbreviations: CGRP, calcitonin gene related peptide; DDC, sodium diethyldithiocarbamate trihydrate; GPx, glutathione peroxidase; LOOM, large for gestational age fetuses from overweight/obese mothers; MS, mercaptosuccinic acid; NAC, N-acetyl cysteine; NOS, nitric oxide synthases; NOX, NADPH oxidase; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase.

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The key vasodilator in placental vascular bed is NO, a free radical synthesized mainly by endothelial NOS (eNOS), whose activity is increased in response to shear stress and, at a lower degree, by vasoactive molecules such as the calcitonin-gene related peptide (CGRP) [10]. Notably NO synthesis is strongly influenced by oxidative stress, which reduces the availability of NO, and negatively regulates the expression and activity of eNOS [11]. Indeed placental exposure to a pro-oxidant such as peroxynitrite increases the perfusion pressure, and reduces the relaxation in response to exogenous NO [12]. Conversely markers of oxidative stress have been reported in IUGR [13] and MO [7,8] placentae, regardless of the fact that maternal BMI and fetal outcomes are substantially different in these two conditions. Thus it is possible to propose that oxidative stress is a common stimuli that negatively modulates endothelial function at short and long term [14] in both IUGR and MO. To address this issue we studied the role of pro- and anti-oxidants on the NOS dependent relaxation in placental chorionic arteries from overweight and/or obese women who delivered LGA babies (LOOM) and IUGR fetuses delivered by women with normal BMI, and samples from normal pregnancies (i.e. normal fetal weight and normal maternal BMI) as controls. Additionally the presence of eNOS, the pro-oxidant enzyme NOX4, and the antioxidant enzymes SOD1, SOD2 and GPx1 in chorionic and umbilical vessels was determined.

2. Materials and methods

2.1. Study participants

Pregnant women attending routine antenatal care at the Maternity of the Hospital Clínico Pontificia Universidad Católica, Santiago, Chile, were invited to participate in the study. The women included in this study were nonsmoking, normotensive, and did not have preeclampsia, pre-gestational or gestational diabetes mellitus and none were on regular medication. Written consent was obtained from those who agreed to participate. The research protocol was approved by the ethics committee of the Faculty of Medicine at the Pontificia Universidad Católica de Chile and by the FONDECYT ethics committee.

In all groups studied gestational age was estimated by ultrasonography before the 12th week of pregnancy. IUGR was defined as body weight below the tenth centile for gestational age and sex according to the national standard weight chart, and the presence of an altered umbilical artery Doppler registry or oligohydramnios. LOOM group was defined as LGA fetuses from mothers that presented overweight or obesity at the first pregnancy control appointment (BMI $\geq$25 before the 12th week of gestation) and maintained this condition until term.

Comparing adequacy of birth weight to gestational age at birth using the National Standard Curve [15] to establish the percentile classification of each new born, all IUGR were ranged between percentile 2 and 10, and LOOM neonates were over 90th centile. Fetal and maternal general characteristics of groups are described in Table 1. Gestational age, ponderal index, maternal age and maternal height were not different between subjects from control, IUGR and LOOM groups. Patients from the IUGR group had a lower mean birth weight and height, compared with control group, whilst these parameters were increased in LOOM subjects. Increased frequency of cesarean deliveries in the IUGR group resulted mainly from fetal non-reassuring state based on antepartum detection of positive contraction stress test, or oligohydramnios along with altered fetal umbilical artery Doppler test. On the other hand maternal weight, pre-pregnancy weight, pre-pregnancy BMI, gestational weight gain, and BMI at term were higher in mothers of LOOM subjects.

Table 1
Fetal and maternal characteristics.

	Control (n = 10)	IUGR (n = 8)	LOOM (n = 8)
Gestational age (weeks)	38.8 ± 0.3	39.1 ± 0.6	39.1 ± 0.2
Birth weight (g)	3372 ± 76	2753 ± 127***	4092 ± 79***
Height (cm)	50.2 ± 0.7	47.7 ± 1.0*	52.4 ± 0.3*
Ponderal index	2.7 ± 0.1	2.5 ± 0.1	2.8 ± 0.1
Gender (F/M)	6/4	4/4	5/3
Delivery (C/V)	3/7	6/2#	2/6
Maternal age (years)	33.7 ± 1.9	30.9 ± 1.9	29.3 ± 1.5
Maternal height (m)	1.60 ± 0.03	1.60 ± 0.03	1.63 ± 0.02
Pre-pregnancy weight (kg)	61.3 ± 3.4	58.2 ± 3.3	78.6 ± 4.4**
Pre-pregnancy BMI	24.2 ± 0.7	22.9 ± 1.0	29.7 ± 1.3**
Gestational weight gain (kg)	8.3 ± 0.8	9.0 ± 1.2	13.3 ± 1.8*
BMI at term	27.9 ± 0.7	26.4 ± 1.0	34.7 ± 1.3***

Ponderal index expressed as birth weight × 100 × height^{-3} (g × cm^{-3}), and maternal body mass index (BMI) expressed as kg × m^{-2}. F and M indicate total number of female or male neonates. C and V indicates the number of cesarean (C) or vaginal (V) deliveries in each group. Values are mean ± S.E.M. or frequency, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ determined by ANOVA, #$p < 0.05$ determined by Chi-squared test.

2.2. Wire myography

Third and fourth order chorionic arteries were dissected from controls (n = 10), IUGR (n = 8) and LOOM (n = 8) being the connective tissue carefully removed. Vessel segments of 2 mm were mounted in a wire-myograph (model 620M; Danish Myo Technology A/S, Aarhus, Denmark), maintained at 37 °C in Krebs buffer (in mmol/L: 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5.5 D-glucose) with constant bubbling (5% CO_2 in air). Isometric force was recorded using PowerLab data acquisition hardware (ADInstruments, Castle Hill, Australia) and LabChart (version 6; ADInstruments) software. After 30 min of equilibration, vessel internal circumferences were determined by measuring the maximal active force in response to KCl (65 mmol/L) as described [16]. This method allows the comparison between different vessels normalizing the vessel tone to similar *in vivo* levels [17]. To address the effect of anti- and pro-oxidant pathways on endothelial function, resting vessels were treated for 20 min with the following agents: a) the antioxidant N-acetyl cysteine (NAC, 10^{-5} M, Sigma–Aldrich); b) the Glutathione peroxidase inhibitor Mercaptosuccinic acid (MS 10^{-3} M, Sigma–Aldrich); c) the Cu/Zn-SOD inhibitor Sodium diethyldithiocarbamate trihydrate (DDC, 10^{-5} M, Sigma–Aldrich) and; d) the pro-oxidant and peroxynitrite donor 3-morpholinosydnonimine (SIN-1, 10^{-6} M). After every incubation, vessels were pre-contracted with KCl and the relaxation in response to cumulative concentrations of calcitonin gene-related peptide (CGRP, 10^{-10}-10^{-6} mol/L) was determined in absence or presence of the antioxidant or inhibitor. All the responses were determined in vessels pre-constricted with 31.2 mmol/L KCl, which evoked the half-maximal contraction in these arteries, and expressed as a percentage of relaxation relative to this effects of KCl (%Kmax) and adjusted to concentration response curves.

2.3. Immunohistochemistry

Umbilical cords and chorionic arteries were washed with cold phosphate buffer saline solution (PBS, in mmol/L: 136 NaCl, 2.7 KCl, 7.8 Na_2HPO_4, 1.5 KH_2PO_4, pH 7.4), dissected in segments of 5 mm, treated overnight with paraformaldehyde (4% in PBS) and included in paraffin. Deparaffinized and rehydrated histological sections of 4 μm were subjected to heat-induced antigen retrieval using 100 mmol/L citrate buffer (pH 6.0) in a steam cooker for 15 min at 95 °C. Samples were treated with 3% H_2O_2 in PBS for 30 min to quench endogenous peroxidase activity. After rinsing in PBS for 5 min, all slides were incubated for 1 h with protein block solution (Cas-Block, Zymed Laboratories, South San Francisco, CA, USA). Sections were incubated overnight at 4 °C with primary anti-eNOS (1:2000) (Santa Cruz), SOD1 (1:1000) (Abcam), SOD2 (1:10) (Abcam), NOX4 (1:500) (Abcam), GPx1 (1:500) (Abcam) and vWF (1:10.000) (Sigma) antibodies. Immunostaining was performed using HRP-conjugated secondary antibodies and binding was determined with NovaRED kit (Vector, Burlingame, CA, USA), treating for 4 min. Slides were counterstained with Harris hematoxylin and permanently mounted. Specificity of the staining was determined by incubation of sections in the absence of the primary antibody. Sections were examined under a IX81-Olympus microscope, and images were captured using a digital camera (Olympus DP-71) and software (Olympus DP-BSW)[16]. At least five slides of every sample were analyzed for each antibody probed in four samples from each group.

2.4. Statistical analysis

Values are mean ± S.E.M., where n indicates the number of placentae from which chorionic arteries were obtained. Comparisons between groups were performed by analysis of variance (ANOVA). If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by Fishers Least Significant Difference test. Data from isolated vessels reactivity were adjusted to dose–response curves from which maximal responses were obtained. Comparison of curves and maximal responses under different conditions were analyzed by ANOVA. All the analyses were carried out with Graphpad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered the cut-off for statistical significance.

3. Results

3.1. Basal relaxation response of chorionic arteries from IUGR and LOOM

Comparison of relaxation in basal conditions showed that CGRP induced a concentration dependent relaxation in control, IUGR and