Placental gene expression of calcitonin gene-related peptide and nitric oxide synthases in preeclampsia: effects of magnesium sulfate

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Abstract. Objective. To determine placental gene expression of calcitonin gene-related peptide (CGRP), calcitonin receptor-like receptor (CRLR), receptor activity modifying protein 1 (RAMP1), and endothelial and inducible nitric oxide synthases (eNOS and iNOS) in mild preeclampsia, and to assess the effects of magnesium sulfate (MgSO\textsubscript{4}). Methods. Term placentas were obtained from 10 normotensive (NT group), 10 preeclamptic (PE) patients treated with 0.9% NaCl solution (PES group), and 8 PE women who received MgSO\textsubscript{4} (PEMgSO\textsubscript{4} group). The levels of mRNA were evaluated by real-time PCR. Results. Placental gene expression of CRLR, RAMP1 and iNOS were significantly higher (p < 0.001) in the PES group than in the NT group, without changes in CGRP. In addition, eNOS expression was 67% lower (p < 0.001) in the PES group. When compared with the PES group, the PEMgSO\textsubscript{4} group showed significantly higher expression (p < 0.05) of CGRP, CRLR and eNOS, while iNOS was significantly lower (p < 0.05). Conclusion. Placental gene expression of CRLR, RAMP1 and iNOS is higher in preeclampsia than in normal pregnancy, and MgSO\textsubscript{4} treatment increased CGRP and CRLR and presented opposite effects upon eNOS and iNOS.

Key words: magnesium sulfate, calcitonin gene-related peptide, nitric oxide synthases, placenta, preeclampsia

Preeclampsia (PE) affects 6-8% of pregnant women and is diagnosed by the simultaneous presence of hypertension and proteinuria [1]. Eclampsia is a rare but serious complication of PE characterized by the onset of generalized seizures, which can be prevented by treatment with magnesium sulfate (MgSO\textsubscript{4}) [2, 3]. In addition to its anticonvulsive effects, MgSO\textsubscript{4} decreases blood pressure [4, 5] through a still unknown mechanism. It is thought that PE originates in the placenta [6], and that high blood pressure in PE may be due to generalized endothelial dysfunction and/or results from an imbalance in the production of vasoactive factors [7].

Regarding vasodilators in PE, we have demonstrated that MgSO\textsubscript{4} treatment increases circulating levels of calcitonin gene-related peptide (CGRP) without changes in nitric oxide (NO) [4]. CGRP as well as two of the three isoenzymes involved in NO production, endothelial and inducible NO synthases (eNOS and iNOS, respectively), have been found in placenta [8-11]; however, there is no report about the effects of MgSO\textsubscript{4} on placental expression of these two isoenzymes and CGRP. Endothelial NOS is a constitutive enzyme whose activity requires calcium/calmodulin, and is predominantly expressed in endothelial cells [12]. The expression of eNOS is downregulated...
by tumor necrosis factor α (TNF-α), a proinflammatory cytokine [13]. In contrast, iNOS is a calcium/calmodulin independent enzyme, and its gene expression is stimulated in cells subjected to proinflammatory cytokines such as TNF-α [14]. CGRP produces vasodilatation through a heterodimer complex formed by a seven transmembrane receptor, calcitonin receptor-like receptor (CRLR) and a receptor activity modifying protein 1 (RAMP1) [15, 16]. During normal pregnancy, CGRP and NO are considered as blood pressure regulator factors in both systemic and placental compartments [17, 18], which may contribute to decrease the vascular resistance established in this physiological state [19]. Since PE is associated with increased vascular resistance, the aim of the present study was to determine whether placental gene expression of CGRP and its heterodimer complex receptor, as well as eNOS and iNOS is altered in this pathological condition, and if MgSO₄ treatment is associated with changes in these vasodilator factors.

Materials and methods

Placental samples
Placentas were collected from patients in accordance with the guidelines of the Declaration of Helsinki, and the study protocol was approved by the Human Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. The study was performed cross-sectionally at term delivery and included placentas obtained from 28 subjects without significant differences in biological and gestational ages: 10 normotensive (NT group) women and 18 mild preeclamptic (PE group) women. Among the PE group, 10 subjects were treated with 0.9% NaCl solution (PES group) and 8 women received MgSO₄ (PEMgSO₄ group) as previously described [20]. Magnesium sulfate treatment consisted of a loading dose of 4 g administered intravenously over a period of 30 minutes followed by a maintenance dose of 1 g per hour for 6 hours [20]. For ethical reasons, all severe preeclamptic subjects received MgSO₄ treatment and no control PES group was available. Thus, only women with mild preeclampsia were included in this study. The diagnosis of mild PE was based on the simultaneous presence of hypertension (systolic blood pressure ≥ 140 mmHg and < 160 mmHg and/or diastolic blood pressure ≥ 90 mmHg and < 110 mmHg) and proteinuria (≥ 30 mg/dL) [1]. Only those women giving birth to a single newborn with Apgar scores of 7-10 were included in the study. Subjects with preexisting hypertension or previous PE, or liver, renal, heart or any other endocrine disorders, including those under nutritional supplements, diuretics, and hormonal treatments, were excluded from the study. Placentas were collected immediately after delivery. Cotyledons, which include syncytiotrophoblast and cytotrophoblast cells as well as blood vessels, were removed from the central part of each placenta, washed repeatedly in 0.9% NaCl to eliminate blood excess, and frozen in liquid nitrogen and stored at -75°C until mRNA expression studies.

Total RNA was isolated from each placental homogenate following the guanidine isothiocyanate and CsCl gradient centrifugation method [21]. RNA integrity was confirmed by ethidium bromide staining after 1.5% agarose gel electrophoresis. RNA concentration was determined by UV-light absorbance at 260 nm (Beckman DU640, Fullerton, CA, USA). Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) was used for reverse transcription (RT) and the reaction was carried out at 55°C for 30 min using 2.5 μg of placent al total RNA in a total volume of 20 μl containing 200 U of the reverse transcriptase, 50 pmol of anchored-oligo(dT)₁₈ primer, 1 mM of each dNTP, and 1x RT buffer (50 mM Tris-HCl, 30 mM KCl and 8 mM MgCl₂, pH 8.5).

Real time polymerase chain reaction (real time PCR)

The mRNA levels of CGRP, CRLR, RAMP1, eNOS and iNOS were quantified by real time PCR using the ABI PRISM 7000 Sequence Detection System (Foster City, CA, USA). Taqman FAM (6-carboxyfluorescein) and VIC dye-labeled probes were obtained from Applied Biosystems (Foster City, CA, USA) and used as fluorescent reporters to detect amplification products. Primers and probes for CGRP, eNOS and iNOS were ordered as kits: Hs00266142_m1, Hs00167166_m1 and Hs00167248_m1 (Assays-on-Demand, ABI, Foster City, CA, USA), whereas primers and probes for CRLR and RAMP1 were designed and purchased from Applied Biosystems (Foster City, CA, USA). Validation of amplification efficiency was made for every primer/probe set and was calculated for each run. Human 18S rRNA (Pre-developed Taqman Assay Reagents) was used as endogenous constitutive gene control to normalize gene expression of each gene studied. Standard curves for each primer/probe were computed from a series of serial template dilutions from 0.187 to 187 ng. PCR was carried out in 96-well plates with
cDNA equivalent to 3.5 ng of total RNA isolated individually from each placental homogenate. Thermal cycling conditions were 10 min at 95°C followed by 50 cycles at 95°C for 1 min, and 60°C for 1 min. Data were collected using the ABI PRISM 7300 SDS analytical thermal cycler (Applied Biosystems). Samples were tested in triplicate. The relative quantification of vasoactive factor gene expression was performed using the comparative threshold cycle method (CT). The CT is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level. Negative controls were included in the reaction plate.

**Statistical analysis**

Results are presented as median with interquartile ranges. Significant differences among groups were determined using the Kruskal-Wallis test, and differences between groups were evaluated using the Mann-Whitney U test. Differences were considered statistically significant at p < 0.05.

As shown in figure 1, CGRP gene expression was significantly different among the NT, PES and PEMgSO4 groups [1.00 (0.97-1.01) vs 0.83 (0.66-1.50) vs 2.50 (1.65-5.15), (p < 0.01), respectively]. Statistical analysis between groups showed that placental expression of this vasodilator was not significantly different between the NT and PES groups. However, a significantly higher expression of CGRP was observed in the PEMgSO4 group as compared with the NT (p < 0.01) and PES (p < 0.01) groups.

Placental gene expression of CRLR was significantly different among the NT, PES and PEMgSO4 groups [1.00 (0.95-1.05) vs 2.22 (1.50-2.44) vs 5.30 (2.57-6.43), (p < 0.0001), respectively]. In addition, placental expression of CRLR was significantly different between the NT and PES (p < 0.001), the NT and PEMgSO4 (p < 0.001), as well as between the PES and PEMgSO4 (p < 0.05) groups (figure 1).

**Figure 1.** Placental mRNA levels of CGRP (upper panel), CRLR (middle panel) and RAMP1 (lower panel) analyzed by real time PCR. Values were normalized by rRNA 18S gene expression. Normotensive (NT, n = 10); preeclamptic (PE) 0.9% NaCl treated group (PES, n = 10) and preeclamptic (PE) MgSO4-treated group (PEMgSO4, n = 8). Data are presented as median with interquartile ranges. Values in groups with different characters are significantly different, p < 0.05.
RAMP1 gene expression was significantly different among the NT, PES and PE MgSO₄ groups [0.99 (0.91-1.03) vs 3.05 (1.70-4.10) vs 3.75 (2.20-4.15), (p < 0.0001), respectively]. RAMP1 placental expression was higher in the PES group than in NT group (p < 0.001). Moreover, the PEMgSO₄ group showed a higher placental expression when compared with the NT group (p < 0.001), but was not significantly different from the PES group (figure 1).

Placental expression of eNOS and iNOS is shown in figure 2. Statistical analysis among the NT, PES and PEMgSO₄ groups showed significant differences of placental expression of eNOS [1.00 (0.97-1.03) vs 0.33 (0.30-0.38) vs 1.30 (1.07-2.17), (p < 0.0001), respectively] and iNOS [0.99 (0.95-1.10) vs 4.89 (3.88-6.46) vs 3.03 (2.00-4.03), (p < 0.0001), respectively]. As compared with the NT group, placental expression of eNOS was significantly lower in the PES group (p < 0.001), and higher in the PEMgSO₄ group (p < 0.05) (figure 2). Regarding iNOS (figure 2), placental expression of this enzyme was significantly higher in the PES and the PEMgSO₄ groups as compared with the NT group (p < 0.001). As compared with the PES group, placental expression of iNOS was significantly lower in the PEMgSO₄ group (p < 0.05).

Discussion

The aim of the present study was to determine whether placental gene expression of CGRP and its heterodimer complex receptor, as well as eNOS and iNOS is altered in preeclampsia, and to evaluate MgSO₄ treatment on the expression of these vasodilator factors.

Results showed that placental expression of CGRP was not altered in mild preeclampsia since similar values were found in the NT and PES groups, which is in accordance with a previous study [22]. In contrast, placental gene expression of CRLR and RAMP1 was significantly higher in the PES group than in the NT group. This overexpression may be explained by the hypoxic state that results from the well known reduced uteroplacental flux seen in preeclampsia [1, 6]. In support of this observation, it has been shown that chronic hypoxia increases CRLR and RAMP1 expression in human vascular smooth muscle cells [23]. Regarding the effects of magnesium sulfate treatment, placental expression of CGRP was significantly higher in the PEMgSO₄ group as compared with PES control group. Interestingly, we have previously observed increased maternal CGRP serum levels in preeclamptic women treated with MgSO₄ [4, 24]. We do not know if the increase in placental CGRP mRNA results in parallel changes in CGRP protein levels. If this parallelism occurs, a high CGRP level may stimulate cAMP synthesis, which in turn increases placental CGRP expression since the gene encoding this peptide contains cAMP response elements (CRE) [25]. In addition, the possible association between MgSO₄ and CGRP expression is supported by the observation that ionic magnesium enhances the binding of CREB to CRE [26]. Regarding CRLR, placental expression of this receptor was also increased in the PEMgSO₄ group. Higher CRLR mRNA could result from the parallel increase in CGRP levels. In this

Figure 2. Placental mRNA levels of eNOS (upper panel) and iNOS (lower panel) analyzed by real time PCR. Values were normalized by rRNA 18S gene expression. Normotensive (NT, n = 10); preeclamptic (PE) 0.9% NaCl treated group (PES, n = 10) and preeclamptic (PE) MgSO₄-treated group (PEMgSO₄, n = 8). Data are presented as median with interquartile ranges. Values in groups with different characters are significantly different, p < 0.05.
regard, it has been shown that CRLR mRNA levels correlate positively with CGRP [27].

In the present study, we also determined placental expression of eNOS and iNOS. Placental NOS was differentially expressed in the absence and presence of MgSO₄. Indeed, placental expression of eNOS was low in the PES group, while that of iNOS was high, and MgSO₄ treatment was associated with opposite effects on the expression of these isoenzymes. Since preeclampsia is associated with high levels of TNF-α [28, 29], this proinflammatory factor may be involved, at least in part, in the differential expression of these isoenzymes. Regarding MgSO₄ treatment, it is probable that MgSO₄ induces changes in the levels of the proinflammatory cytokines, which may contribute to the opposite effects of this treatment upon NOS expression. This possibility is supported by the fact that perfusion of MgSO₄ to isolated human placental cotyledons completely inhibited the increase of IL-1β secretion induced by angiotensin II [30], whereas low magnesium has been associated with an enhanced release of IL-1β and TNFα from rat alveolar macrophages in culture [31]. All these observations deserve to be further investigated.

In summary, the results of the present study showed that placental gene expression of CRLR, RAMP1 and iNOS is increased in preeclampsia, and demonstrated that MgSO₄ treatment is associated with increased CGRP and CRLR and presents opposite effects on eNOS and iNOS placental expression.

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References


