Differential expression of endocannabinoid system in normal and preeclamptic placentas: Effects on nitric oxide synthesis

C. Abána,*, G.F. Leguizamónb, M. Cellab, A. Damianoc, A.M. Franchib, M.G. Farinaca

aLaboratorio de Fisiopatología Placentaria, Centro de Estudios Farmacológicos y Botánicos (CEFYBO, CONICET), Facultad de Medicina, Universidad de Buenos Aires (UBA), Paraguay 2155, C1121ABG Buenos Aires, Argentina
bLaboratorio de Fisiopatología de la Preñez y el Parto, (CEFYBO, CONICET), Facultad de Medicina, Universidad de Buenos Aires (UBA), Argentina
cLaboratorio de Biología de la Reproducción, Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (UBA), Argentina
dCentro de Educación Médica e Investigaciones Clínicas (CEMIC), Unidad de Embarazo de Alto Riesgo, Departamento de Obstetricia y Ginecología, Argentina

ABSTRACT

Anandamide (AEA) is a lipid mediator that participates in the regulation of several reproductive functions. This study investigated the endocannabinoid system in normal (NP) and preeclamptic (PE) placentas, and analyzed the potential functional role of AEA in the regulation of nitric oxide synthesis.

The protein expression and localization of NAPE-PLD, FAAH and CB1 receptor were analyzed in normal and preeclamptic pregnancies using immunoblotting and immunohistochemistry. NAPE-PLD expression was shown to be significantly higher \( (p < 0.05) \) in PE tissues than in NP. In contrast, a decrease in FAAH protein \( (p < 0.001) \) was detected in placentas collected from women with preeclampsia. Both enzymes were mainly located in the syncytiotrophoblasts from normal and preeclamptic tissues. No differences were seen in CB1 receptor from both groups of placental villous.

Exogenous and endogenous AEA significantly increased NOS activity. Although pre-incubation with AM251 (CB1 antagonist) had no effect, co-incubation with both AEA and AM251 diminished NOS activity from normal term placentas. We observed increased NOS activity in placentas from women with preeclampsia compared with normotensive pregnant women. Furthermore, NOS activity from preeclamptic tissues was diminished by co-treatment with AM251, illustrating that the NO levels could be modulated by AEA.

These data suggest that AEA may be one of the factors involved in the regulation of NOS activity in normal and preeclamptic placental villous. Interestingly, the differential expression of NAPE-PLD and FAAH suggests that AEA could play an important role in the pathophysiology of PE.

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1. Introduction

Cannabis use during pregnancy has been associated with increased risk adverse birth outcomes [1] suggesting that the endogenous ligand of cannabinoid receptors would participate of reproduction.

Endocannabinoids are an important family of lipid-signaling molecules that act as endogenous ligands of cannabinoid receptors (CB1 and CB2) [2], with anandamide (N-arachidonoylthanolamine, AEA) being the first agonist identified [3]. AEA is synthesized on demand by different pathways, mainly from membrane bound N-arachidonoyl phosphatidylethanolamine (NAPE) through cleavage by a phospholipase D (NAPE-PLD) [4]. Its effects finish by the uptake of AEA into the cell followed by a rapid degradation by the enzyme fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine [5]. CB1 and FAAH have been localized in human term placenta tissue [6], and several studies suggest that FAAH protein is crucial for the control of circulating AEA concentrations [7,8].

Anandamide has been reported to have pleiotropic effects on reproduction; however, the mechanism by which it exerts these effects is unclear. Higher AEA concentrations were found in the placenta [9], and significant changes in AEA levels have been detected at the end of pregnancy in maternal blood [10], suggesting that the endocannabinoid system could modulate physiological functions during pregnancy and labor.

Nitric oxide (NO) is a potent local vasodepressor that has been shown to play a central role in the physiological regulation of hemodynamic flow, thus contributing to the maintenance of low vascular resistance in the feto-placental circulation [11]. NO is synthesized by the action of NO synthases (NOS), and the presence
of both constitutive and inducible isoforms has been demonstrated in human trophoblast [12,13].

The role of NO seems to support the low basal tone in the fetoplacental circulation keeping placental vessels in a vasodilated state and attenuating the effects of vasoconstrictors [14].

Several alterations of NO synthesis have been described in pregnancies complicated by intrauterine growth restriction or preeclampsia (PE) [15]. The latter is one of the leading causes of maternal and neonatal adverse outcomes. It is characterized by hypertension and proteinuria after 20 weeks of gestation in previously normotensive women [16]. It is clear that vascular endothelial dysfunction is an important component of this disorder. However, abnormal placentation/reduced placental perfusion occurring in the first trimester (Stage 1) is not always sufficient; and requires interaction with genetic, behavioral or environmental factors to lead to the development of clinical preeclampsia (Stage 2) [17].

It was previously reported that aberrant endocannabinoid signaling confers premature trophoblast stem cell differentiation, defective trophoblast development and invasion [18]. Nevertheless, to date there are no reports of a direct relationship between increased levels of AEA and preeclampsia.

In PE, NO activity regulation is still unclear. Different studies have shown that NOS activity either decreased, increased or remained unchanged compared to normal placenta [19–21].

Over the last years, we and other researchers have shown that AEA is able to modulate NO synthesis [22–24]. However, the effect of AEA on nitric oxide synthesis in human placenta has not been examined.

The aim of this study was to determine whether PE, a state of placental abnormality, is associated with alterations in the endocannabinoid system, and to investigate whether AEA could modulate nitric oxide synthesis in normal and preeclamptic placentas.

2. Materials and methods

2.1. Drugs and chemicals

Aminoguanidine (AG), L-valine, secondary horse-radish peroxidase (HRP)- conjugated and anti-ß-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [14C]-Arginine was from Amersham Corp. (Arlington Heights, IL, USA). AM251 was purchased from Tocris Cookson Inc. (Ellisville, MO, USA). [3H]-anandamide (specific activity 172.4 Ci/mmol) was provided by Perkin Elmer (Boston, MA, USA). TLC aluminum Silica Gel plates were purchased from Merk KGaA (Darmstadt, Germany). The NAPE-PLD antibody and URB-597 were purchased to Kenneth Mackie. The anti-FAAH antibody was a gift from Dr. Benjamin Cravatt.

2.2. Placental tissues

Dowex AG 50W-X8 cation exchange resin and the Western blot reagents were obtained by the modiﬁed method of Bredt & Snyder [26] which measures the conversion of [14C]-ß-arginine into [14C]-citrulline. Briefly, tissues were homogenized in a buffer containing 20 mM HEPES, 4.5 μM CaCl2, 100 mM DTT, 0.12 mM NADPH and 25 mM valine. After homogenization, 10 μM [14C]-ß-arginine (0.3 μCi) was added and samples were incubated for 15 min in a 5% CO2 atmosphere at 37 °C and immediately centrifuged 3000 g at 4 °C for 10 min. The supernatant was applied to a 1 ml DOWEX AG 50W-X8 columns (Na+ form) equilibrated with HEPES medium and 1[14C]-citrulline was eluted in 3 ml of water. The radioactivity was measured by liquid scintillation counting. Protein concentration was measured by the Bradford assay [23]. Enzyme activity is reported as fmol of [14C]-citrulline produced by mg of protein during 15 min.

2.4. Determination of fatty acid amide hydrolase (FAAH) activity

FAAH activity was assayed as described by Pania et al. [27] with minor modifications. Briefly, explants from NP and PE placentas were homogenized in buffer Tris pH 7.6 (20 mM Tris/HCl, 1 mM EDTA) and 100 μg of protein were incubated for 10 min at 37 °C in 200 μl of buffer Tris pH > 8.5 (50 mM Tris/HCl) containing 100 μM [3H]-anandamide (0.05 μCi). The reaction was stopped by the addition of a chloroform: methanol (1:1 v/v) mixture. Samples and authentic arachidonic acid (AA) standard were applied on TLC aluminum Silica Gel 60 sheets with concentration factor 400. The sample was measured by the Bradford method [25] and 100 μg of protein were loaded in each lane. Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were incubated with anti-NAPE-PLD (1:1000), anti-FAAH (1:150), or anti-CB1 (1:250) followed by anti-ß-actin (1:4000). Blots were washed and incubated with horse-radish peroxidase-conjugated anti-ß-actin IgG as the secondary antibody and developed by ECL. The intensity of bands determined using the Image J (NIH) program.

2.5. Determination of NO activity

Oxynitrites were quantified by the modified method of Bredt & Snyder [26] which measures the conversion of [14C]-ß-arginine into [14C]-citrulline. Briefly, tissues were homogenized in a buffer containing 20 mM HEPES, 4.5 μM CaCl2, 100 mM DTT, 0.12 mM NADPH and 25 mM valine. After homogenization, 10 μM [14C]-ß-arginine (0.3 μCi) was added and samples were incubated for 15 min in a 5% CO2 atmosphere at 37 °C and immediately centrifuged 3000 g at 4 °C for 10 min. The supernatant was applied to a 1 ml DOWEX AG 50W-X8 columns (Na+ form) equilibrated with HEPES medium and 1[14C]-citrulline was eluted in 3 ml of water. The radioactivity was measured by liquid scintillation counting. Protein concentration was measured by the Bradford assay [23]. Enzyme activity is reported as fmol of [14C]-citrulline produced by mg of protein during 15 min.

2.6. Immunohistochemistry

Normal and PE tissues were fixed overnight in 10% formaldehyde 0.1 mol/L sodium phosphate buffer (PBS), pH 7.4, dehydrated, and embedded in paraffin as have previously described in Ref. [28]. Tissue slices (4–5 μm) were incubated in 3% hydrogen peroxide (H2O2) methanol to block endogenous peroxidase. Nonspecific binding sites were blocked by incubation in blocking reagent (DAKO LSAB kit, Dako Corp) for 30 min. Then, tissues slices were incubated overnight (4 °C) with anti-NAPE-PLD (1:200), anti-CB1 (1:50) or anti-FAAH (1:50). Samples were placed in prediluted link antibody and incubated in a solution of streptavidin conjugated

<table>
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<th>Table 1</th>
<th>Normal placentas</th>
<th>Preeclamptic placentas</th>
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<tr>
<td>Number of pregnant women</td>
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<td>14</td>
</tr>
<tr>
<td>Gestational age, yr</td>
<td>24.6 ± 5.1</td>
<td>26.7 ± 6.3</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>3320 ± 540</td>
<td>2580 ± 1130</td>
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<tr>
<td>Proteinuria</td>
<td>Negative</td>
<td>++</td>
</tr>
<tr>
<td>Systolic</td>
<td>115.0 ± 3.9</td>
<td>163.0 ± 4.5*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>62.1 ± 2.3</td>
<td>113.0 ± 2.0**</td>
</tr>
</tbody>
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*P < 0.01. **P < 0.001. Values are means ± Standard Deviation. LMP: last menstrual period.
Fig. 1. Placental NAPE-PLD and FAAH protein expression in normal and preeclamptic tissues. Representative Western immunoblotting showing NAPE-PLD (A) and FAAH (C) expression in placental villous from normal and preeclamptic pregnancies. Graphs show densitometric analysis performed and the band corresponding to each protein was normalized to β-actin. Data are expressed as the mean ± SEM from normal (n = 10) or preeclamptic tissues (n = 10). Variations in NAPE-PLD and FAAH expressions denote statistically significant (Student’s t-test, *p < 0.05 and ***p < 0.001). Immunohistochemical staining for NAPE-PLD (B) and FAAH (D) was performed in three patients from each group and representative sections of placental villous from normal pregnant women and preeclamptic patients shown at magnification ×1000. Arrows indicate immunopositive syncytiotrophoblast layer. In both, non-immune rabbit serum without the primary antibody was used as a negative control. FAAH activity in normal and preeclamptic tissues (E). Graph shows FAAH activity in normal (n = 6) and preeclamptic (n = 6) villous. Bars represent means ± SEM (Student’s t-test, *p < 0.05 vs. normal placenta).
horse-radish peroxidase. Staining achieved with Vectastain kit (Vector Laboratories) and labeling was visualized by reaction with DAB (Diaminobenzidine tetrahydro-chloride) and counterstained with hematoxylin. Non-immune rabbit serum without the primary antibody was used as a negative control. Sections were viewed and photographed under a Nikon Eclipse E200 microscope.

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software (San Diego, CA, USA). Comparisons between values of different treatment were performed using one-way analysis of variance (ANOVA). Significance was determined using Tukey’s multiple comparison tests for unequal replicates or Student’s t-tests. The results represent mean ± SEM of three independent experiments performed. Differences between means were considered significant when p < 0.05.

3. Results

3.1. Expression of NAPE-PLD and FAAH proteins in normal and preeclamptic placentas

The relevant biosynthetic pathway for AEA involves the activity of a NAPE-PLD [4]. This enzyme was proposed as a crucial determinant of AEA levels [29]. Therefore, we decided to evaluate by Western blot the NAPE-PLD protein expression in normal and preeclamptic tissues. Representative results are presented in Fig. 1A. Interestingly, quantitative densitometric analysis of protein bands obtained for NAPE-PLD was significantly enhanced in samples from women with preeclampsia (p < 0.05) when compared to normal tissues. Specific NAPE-PLD immunoreactivity revealed a labeling localized in the apical membrane of syncytiotrophoblast from both normal and preeclamptic placentas (Fig. 1B).

Our result showed an immunoreactive band of 63 kDa coincident with FAAH in normal placentas, while protein expression was lower or undetectable in samples from women with preeclampsia (Fig. 1C). Densitometric analysis detected a significant decrease (p < 0.001) in FAAH protein levels in PE.

We confirmed by immunohistochemistry that FAAH was mainly located in the apical membrane of syncytiotrophoblast in normal placentas but weakly detected in some villi from PE tissues (Fig. 1D).

It is well known that AEA levels are principally regulated by FAAH [30], but its activity has not been evaluated in human term placentas. Our results show that, compared with controls, placentas from women who developed preeclampsia had significantly reduced FAAH activity (Fig. 1E).

3.2. Effects of AEA on placental NOS activity

We have previously reported that AEA modulate NOS activity in rat placenta [22]. In order to determine the effects of AEA on NOS activity in normal human placentas (NP), a concentration-response curve of NOS activity was carried out. We observed that AEA (10 nM or 100 nM) significantly increased NOS activity in normal placentas (Fig. 2A).

To study the effects of endogenous AEA, explants from NP were pre-incubated with URB-597, a selective FAAH inhibitor. We found that the reduction of AEA hydrolysis significantly (p < 0.01) increased placental NOS activity (Fig. 2B).

Consistent with the exogenous AEA effects, this finding suggested that changes in AEA levels modified NO production in human term placentas.

3.3. Expression and localization of CB1 receptor in normal and preeclamptic placentas

Previously, studies have clearly demonstrated very dense labellings of CB1 in villi from non-laboring women [6,31], but protein expression and localization of this receptor has not been evaluated in preeclamptic placentas.

By Western blot analysis we detected a band of approximately 58 kDa in all samples analyzed from normal and PE tissues (Fig. 3A). Quantitative densitometric analysis revealed similar protein expression.

We analyzed the localization of CB1 in chorionic villi of patients with preeclampsia. An analogous CB1 immunoreactivity was found in the apical membrane of syncytiotrophoblast from normal and PE tissues (Fig. 3B).

3.4. Participation of CB1 receptor in AEA effects on NOS activity

Since AEA induced NO production in a separate series of experiments, we studied the participation of CB1 receptor in the effects of AEA on NOS activity. Villi from normal placentas were
pre-incubated with AM251 (CB1 antagonist). Subsequently, AEA was added (10^{-7} M) and NOS activity was measured as described above. While pre-incubation with AM251 had no effect, AEA increased NOS activity through binding to CB1 receptor in human term placentas (Fig. 3C).

3.5. NOS activity in preeclampsia

To date there is great controversy about the role of NO in the pathophysiology of preeclampsia. While some authors suggested that a reduction in NO levels is one of the factors characteristic of this disease [19,32], others found that NOS activity or its metabolites are enhanced in this condition [33]. Here, NOS activity observed in chorionic villi from women with PE was higher (p < 0.05) than NP (Fig. 4A).

Since in PE we observed impaired FAAH protein expression and activity, we speculated that the levels of AEA could be augmented in this entity. To study the participation of endogenous AEA in the levels of NO, we analyzed the effect of AM251 in villi from preeclamptic women. The result (Fig. 4B), strongly suggests that AEA could be one of the factors responsible for the NOS activity observed in PE placentas.

**Fig. 3.** Placental CB1 in normal and preeclamptic tissues. (A) Representative Western blot for CB1 expression from normal and preeclamptic villous tissues. Graphs show densitometric analysis of CB1 expression normalized to β-actin. Data are expressed as the mean values relative to samples from normal (n = 10) or preeclamptic tissues (n = 10). (B) Immunohistochemical staining for CB1 in representative sections of placental villous from normal pregnant women and preeclamptic patients shown at magnification ×1000. Arrows indicate immunopositive syncytiotrophoblast layer. Non-immune rabbit serum without the primary antibody was used as a negative control. (C) Participation of CB1 receptor in AEA effect on NOS activity. Normal placentas (NP) were pre-incubated with AM251 (10^{-7} M), then AEA was added and 30 min latter NOS activity was measured. Values are expressed as means ± SEM. (n = 8). Comparisons between values were performed using ANOVA, ***p < 0.001 vs. NP.
specific phospholipase D (NAPE-PLD), which release AEA and phosphatidic acid [34]. In our research, NAPE-PLD protein levels were higher in placentas obtained from preeclampsia than in those from normotensive women. This results suggest that AEA is produced by human placenta in both physiological and pathological conditions.

Previous reports have shown that high plasma levels of AEA seriously interfere in the progression of pregnancy [35,36]. Local AEA levels depend on its synthesis (mainly by NAPE-PLD) and its metabolism inside the cell by the FAAH enzyme.

In the first trimester, Helliwell et al. [37] supported up-regulation of placental FAAH expression, while Habayeb et al. [38] suggested that even though their distribution was modified, no change in trophoblastic FAAH levels were observed.

On the other hand, while FAAH expression was visible mostly in the syncytiotrophoblast layer [6], another studies reported that FAAH disappears in normal term placentas [31]. Our assays showed that FAAH was localized in syncytiotrophoblast from healthy women without labor and protein expression was detectable in this placentas. Controversies about these observations may arise due to methodological variations (different antibody and sample preparation). Since the placenta is an efficient and selective barrier between maternal and fetal compartments, high FAAH expression in normal placentas could contribute to a decrease in AEA levels in term pregnancies. Additionally, we investigated FAAH protein expression and localization in pathological conditions and found weakened protein levels and immunoreactivity in preeclamptic placentas, compared with normal tissues. Furthermore, we demonstrated for the first time a significant activity in normal human placentas. However, and in agreement with Western blot results, we observed a lower activity of this metabolizing enzyme in preeclamptic tissues. Taken together, these findings suggest that AEA levels could be increased in pathological conditions, and the fetus may be exposed to dangerous levels of this endocannabinoid.

Numerous studies have showed direct effects of steroids, hormones [39], and cytokines [40] on elements of endocannabinoid system. In this context, it has been found that FAAH expression is regulated by the Th1 and Th2 cytokines: IL-4 and IL-10 enhance FAAH activity, whereas IL-2 and INF-γ reduce FAAH expression. Nevertheless, the mechanisms by which NAPE-PLD and FAAH protein expression and activity can be regulated in preeclamptic placentas are still unknown.

Several groups have reported a vasodilatory effect of anandamide through the release of various endothelium-derived releasing factors [41,42]. In the present study we showed that AEA increased placental NOS activity in human term placentas. Interestingly, Marczyno et al. [9] reported that delays in tissue processing cause an important increase in AEA concentration. Therefore, we study placentas treated within 1h after removal of tissues from –80 °C storage. Our assays showed that URB-597, a selective FAAH inhibitor, significantly increased NO synthesis in term placental tissues. These data are consistent with the notion that FAAH was expressed in normal human term placentas.

Placental villi from women delivering at term by elective (non-laboring) cesarean section is characterized by higher CB1 expression than villi from term laboring placentas [31]. Our assays showed that CB1 receptor was detected in syncytiotrophoblast from normal placentas, and its pharmacological blockade with AM251 abolished the effect of AEA on NOS activity. These data indicate that AEA may be an endogenous compound that stimulates CB1 receptor, thus leading to enhancement of NOS activity in human placenta.

Vascular tone is regulated by local factors, and NO levels are considered as one of the essential components. Several reports have demonstrated that NOS are expressed in both normal [12,13] and preeclamptic tissues [43], and have investigated the participation of

![Figure 4](image.png)

**Figure 4.** Participation of AEA on NOS activity in preeclamptic tissues. (A) Graph shown NOS activity in villous tissues from women undergoing cesarean section (n = 8) or preeclamptic patients (n = 8). Values are expressed as means ± SEM. Comparisons between values were performed using Student's t-test, *p < 0.05 vs. NP. (B) Effect of endogenous AEA on NOS activity in preeclamptic tissues. Villous from preeclamptic women (PE) were pre-incubated with AM251 (10⁻⁷ M) and NOS activity was measured. Values are expressed as means ± SEM. (n = 8). Comparisons between values were performed using ANOVA, *p < 0.05 vs. NP.

4. Discussion

Although the expression of the endocannabinoid system has been demonstrated in human placenta, its role in the physiology of this tissue has not been under investigation.

In the present study we demonstrated that some of the components of endocannabinoid system are impaired in preeclamptic placentas. In addition, we found that changes in AEA levels could be some of the factors involved in the regulation of placental NOS activity in both healthy and preeclamptic women.

The primary source for AEA synthesis is an N-arachidonoyl phosphatidylethanolamine (NAPE) through the enzyme NAPE-
NO in the pathophysiology of preeclampsia. Nevertheless, the results obtained were controversial, since some authors demonstrated higher levels of NO in preeclamptic placentas [33], while others detected no changes [44], or found a reduction in placental NO levels [32]. Thus, our findings show high NO activity in choriocarcinoma of trophoblast cell lines and placenta. The discrepancy in the results obtained by different research groups on the expression and activity of NOS between normal and preeclamptic placentas could be provoked by different pharmacological treatments that are given to patients in at different health centers or with different severity of preeclampsia, e.g. nifedipine treated (which can alter NO levels) [45].

In summary, in the present work we found a differential expression in some of the endocannabinoid system components, which could contribute to alterations in the placental levels of AEA in preeclamptic tissues. Additionally we observed that AEA increase NOS activity throughout CB1 receptor in normal and preeclamptic placentas. Thus, we propose that changes in AEA levels generated in preeclampsic tissues due to the sharp decrease in FAAH activity and expression might be contributing to the fine regulation of NOS activity observed in some samples of this entity.

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