Evaluation of the endocannabinoid system in preeclampsia

Ph.D. Thesis

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Budapest
2015

Introduction

Despite great efforts put into the research of preeclampsia, a serious pregnancy-associated illness characterized by high blood pressure and proteinuria developing in the second half of gestation, its exact patomechanism remains unknown. In developed countries, preeclampsia is still responsible for nearly 15% of maternal deaths, with a worldwide incidence of 3-8%. The emergence of the syndrome is linked to the presence of the placenta. Literature describes preeclampsia with the two-stage model: stage 1 consists from abnormal placental development, leading to hypoxia and oxidative stress through impaired uteroplacental circulation, which cause general endothelial dysfunction (stage 2) responsible for the appearance of clinical symptoms. The development of preeclampsia is influenced by both genetic and environmental risk factors, suggesting its multifactorial inheritance.

Failed remodelling of the maternal spiral arteries plays a central role in the pathogenesis of preeclampsia. Extravillous cytotrophoblasts, responsible for the process, are not able to penetrate the wall of the spiral arteries, limiting the remodelling to the superficial layers of the decidua, while myometrial segments of the vessel stay narrow. Suffering in oxidative stress from consequential hypoxia, the placenta releases an abnormal amount of anti-angiogenic factors and trophoblast debris into the maternal circulation, causing the clinical syndrome. The most examined anti-angiogenic factor in preeclampsia pathogenesis, soluble fms-like tyrosine kinase-1 (sFlt-1), is produced from the vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) receptor Flt-1 *via* alternative splicing mechanism, loosing its transmembrane and signaling domains. The soluble protein takes effect by binding circulating VEGF and PlGF molecules. Due to abnormal placental expression, serum sFlt-1 levels are elevated in preeclampsia, while pro-angiogenic factors VEGF and PlGF serum levels are decreased. This pathologic shift in angiogenic factor balance is detectable several weeks before clinical signs appear, thus having predictive value regarding preeclampsia.

Endocannabinoids (eCBs) are endogenous lipid mediators having similar effects to Δ^9-tetrahydrocannabinol, the main component of *Cannabis sativa*. Their most well-known and researched members are anandamide (N-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG). The ligands exert their effects mainly on the heptahelical, G-protein-coupled cannabinoid receptors CB1 and CB2 *via* inhibition of

adenylate-cyclase, thus reducing cAMP levels. The main enzyme responsible for degradation is the fatty acid amide hydrolase (FAAH) for anandamide, and a specific monoacyl-glycerol-lipase (MAGL) for 2-AG. Endocannabinoids are linked to various physiological and pathological processes of the human body. As for the cardiovascular system, their effects manifest in reducing blood pressure and myocardial contractility, mediated by CB1 receptors localized primarily in the myocardium, blood vessel walls, central and autonomic nervous system. Hypotensive effects prevail through various, tissue- and species-specific pathways, including CB1, transient receptor potential vanilloid 1 (TRPV1), nitric-oxide-dependent and –independent mechanisms.

Endocannabinoids play an important role in almost all key steps of the reproductive process. CB1 and CB2 receptors were identified in all phases of oocyte maturation in mammals. ECBs thought to play a key function in oviductal transport: in CB1-knockout mice the blastocyst was trapped longer in the fallopian tube, also lower CB1 transcription levels were measured in women with ectopic pregnancies. CB1 receptor activation inhibited decidualization in human cells *in vitro*, probably due to cAMP-dependent apoptotic processes. Decreased local anandamide concentration improves uterus receptivity for the blastocyst, while high concentrations are embryotoxic and cause impaired trophoblast proliferation and implantation failure. Elevated 2-AG *in vitro* disturbes cytotrophoblast functional and morphological differentiation into syncytiotrophoblasts – this observation confirmes a crucial role of the endocannabinoid system in placentation. Research showed correlation between anandamide levels and onset of labour: higher plasma anandamide concentrations and CB1 expression were measured in women in labour compared to ones not in labour. This suggests that elevated anandamide levels and low CB1 expression are necessary for timely onset of labour.

Objectives

1. The endocannabinoid system plays a central role in female reproduction, including implantation, decidualization and placentation. Several studies showed close correlation between placental and peripheral anandamide levels and spontaneous miscarriage and ectopic pregnancy, also there is a possible role for anandamide in blood pressure regulation. Based on these findings, in our study we aimed to analyze the connection between anandamide levels and preeclampsia. We determined serum anandamide, sFlt-1 and PlGF concentrations of healthy pregnant and preeclamptic women. We also compared the measured anandamide concentrations to relevant clinical characteristics of the study participants.
2. Poor placentation plays a key role in the pathogenesis of preeclampsia, which provides a basis for local examination of the endocannabinoid system. Therefore, besides serum anandamide levels we determined the expressions of further members of the endocannabinoid system, including CB1 and CB2 receptors, and the FAAH enzyme in placental samples from healthy pregnants and preeclamptic women.
3. Earlier studies described the role of local endocannabinoid effect on uterus receptivity and on regulation of trophoblast cell invasiveness. Enhanced local cannabinoid activity (high anandamide level) may lead to impaired placentation, thus causing preeclampsia. Based on the latter, besides determining tissue expression, we examined CB1, CB2 and FAAH localization in placental samples from healthy pregnants and preeclamptic women.

Patients and methods

Study participants

Forty-three preeclamptic patients and 71 normotensive, healthy pregnant women with uncomplicated pregnancies were involved in this case-control study. The study participants were enrolled in the First Department of Obstetrics and Gynecology, at the Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. The preeclamptic patients and healthy pregnant women were matched on the basis of maternal age and gestational age at blood draw, and they were selected accordingly from a larger cohort of preeclamptic patients and healthy pregnant women. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The women were fasting, none were in active labor, and none had rupture of amniotic membranes.

Eighteen preeclamptic patients and 18 normotensive, healthy pregnant women with uncomplicated pregnancies were involved in determination of the CB1, CB2 and FAAH expression and localization. All other criteria were identical to those discussed in the preceding paragraph.

Preeclampsia was defined by increased blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 occasions at least 6 hours apart) that occurred after the 20^{th} week of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24h or ≥1 + on dipstick in the absence of urinary tract infection). Blood pressure returned to normal by the 12^{th} postpartum week in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure ≥160 mmHg systolic or ≥110 mmHg diastolic, or proteinuria ≥5 g/24h (or ≥3 + on dipstick). Early onset of preeclampsia was defined as onset of the disease before the 34^{th} week of gestation (between the 20^{th} and 33^{rd} completed gestational weeks). Fetal growth restriction was diagnosed if the fetal birth weight was below the 10^{th} percentile for gestational age and gender, based on Hungarian birth weight percentiles.

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University, and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

Determination of serum concentrations

Maternal blood samples were obtained from an antecubital vein into plain tubes (with no additive) and centrifuged at room temperature with a relative centrifugal force of 3000 *g* for 10 minutes. The aliquots of serum were stored at -80 °C until the measurements.

Serum anandamide concentrations were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS) technique. Serum samples were supplemented with internal standard solution (100 ng/ml D_4-anandamide), and analytes were extracted with acetonitrile. The clear supernatant was evaporated in N_2, and re-dissolved samples were analyzed by HPLC-MS technique. The quantification was carried out with calibration curve, calibration points were obtained from blank serum samples spiked with different concentration of anandamide. The calibration range was between 0.1 and 100 ng/ml anandamide. HPLC system consisted of a Jasco X-LC binary pump and autosampler. The quantification was performed using TSQ Quantum Discovery triple quadrupole mass spectrometer with electrospray ionization source operated in positive ion mode. Analytes were separated using reversed-phase liquid chromatography on a Merck Purospher Star C18 column with gradient elution. (Mobile phases were 100 mM ammonium-acetate (0.1% formic acid) and methanol at a flow rate of 300 µl/min.)

Serum total soluble fms-like tyrosine kinase-1 (sFlt-1) and biologically active placental growth factor (PlGF) levels were measured by electrochemiluminescence immunoassay (Elecsys, Roche, Mannheim, Germany, Cat. No. 05109523 and 05144671, respectively) on a Cobas e 411 analyzer (Roche, Mannheim, Germany).

Determination of serum sFlt-1 is based on sandwich principle. Total duration of the assay is 18 minutes. 1st incubation: 20 µl of sample, a biotinylated monoclonal sFlt-1-specific antibody, and a monoclonal sFlt-1-specific antibody labeled with a ruthenium

complex react to form a sandwich complex. 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Serum PlGF concentrations were measured using the same sandwich principle described before, the total duration of assay was 18 minutes. 1st incubation: 50 µL of sample, a biotinylated monoclonal PlGF-specific antibody, and a monoclonal PlGF-specific antibody labeled with a ruthenium complex react to form a sandwich complex. Following parts of the measurement are identical to those discussed in the preceding paragraph.

Western blot and immunohistochemistry

The Western blot was performed on full-thickness blocks from the chorionic plate to the basal plate in order to determine the overall expression of CB1, CB2 and FAAH in the placenta. One g of placental tissue was minced well and homogenized in 10 ml of lysis buffer (20mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 2 mM DTT, 1 mM Na_3VO_4, 25 µg/ml PMSF, 2.5 µg/ml Leupeptin, 2.5 µg/ml Aprotinin, 625 µM sodium-pyrophosphate, 1mM β-glycerophosphate, 0,1% Triton) by a homogenizer for 3 x 15 sec. The homogenate was centrifuged at 800 *g* for 15 min at 4 °C, and the pellet was discarded. The supernatant was collected and stored at -80 °C and used within four weeks for assays. To assess FAAH (AT1983a, mouse monoclonal antibody, Abgent Inc., San Diego, CA, USA), CB1 (EB06945 goat polyclonal anti-CB1 antibody, Everest Biotech, England) and CB2 (EB06946 goat polyclonal anti-CB2 antibody, Everest Biotech, England) expressions in human placenta, 60 µg of extracted proteins were used for Western blot analysis.

Samples were prepared in 2x Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 15 minutes. Protein (60 μg) was separated on a SDS-PAGE (9 %) gel followed by a wet transfer to a nitrocellulose membrane for 90 minutes. We used Ponceau S to determine whether proteins migrated uniformly onto the nitrocellulose membrane. After gently rinsing, the membranes were blocked for 1 h at room temperature in 10% (wt/vol) non-fat dried milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and then incubated overnight with antibody against the CB1 or CB2 or FAAH, respectively. The antibodies were diluted (CB1 1:1000, CB2 and FAAH 1:500) in 1% bovine serum albumin in TBST. Blots were incubated in a HRP-conjugated secondary antibody in TBST for 1 h at room temperature and visualized by ECL-Western blotting detection system (Amersham Pharmacia Biotech, England). Mouse testicular homogenate and color molecular weight markers were run parallel with the samples and were used to identify the specific bands. The membrane was stripped at 60 °C for 30 minutes in stripping buffer (100 mM 2-mercaptoetanol, 2% SDS and 62.5 mM TRIS-HCl, pH 7.6), and were reprobed with MAPK1 antibody (1:1000) to normalize for loading.

Western blot signals were semi-quantified by densitometry analysis using a GELDOC 1.00-UV system (Biorad, Hercules, CA, USA). The signals of the specific bands in densitometry unit were adjusted according to the changes of the corresponding density of MAPK1 bands on the same loaded membrane. The deviation of the density of MAPK1 bands from the mean was used to normalize the value of the CB1, CB2 or FAAH bands. The corrected signals of the preeclamptic placentas were expressed as % of the mean values of normal placentas.

Immunohistochemical staining was performed on 16 placental samples from each study group. Anti-CB1 (GTX100219) and anti-CB2 (GTX101357) rabbit polyclonal antibodies were obtained from GeneTex (Irvine, CA, USA), whereas anti-FAAH (AT1983a) mouse monoclonal antibody was purchased from Abgent (San Diego, CA, USA). In comparison to Western blot, different CB-antibodies were chosen due to the incompatibility of the antibodies used during Western blot with IHC technology. Tissue sections (3 μm thick) were mounted onto SuperFrost Ultra Plus Adhesion Slides (Thermo Scientific, USA), dried in thermostat at 56 °C for 1 h, then 24 h at room temperature before use. Immunostaining process was carried out with Leica

BOND-MAX fully automated IHC & ISH system (Leica Biosystems, St. Louis, MO, USA), using Bond Polimer Refine Detection kit (Leica Biosystems), which included peroxide block (3% Hydrogen peroxide), post primary polymer penetration enhancer (10% animal serum in Tris-buffer saline and 0.09% ProClin™ 950), polymer Poly-HRP anti-mouse/rabbit IgG (each at 8 μg/ml, containing 10% animal serum in Tris-buffer saline and 0.09% ProClin™ 950), DAB Part 1 (66 mM 3,3'-Diaminobenzidine tetrahydrochloride, in stabilizer solution), DAB Part B (0.05% Hydrogen Peroxide in a stabilizer solution) and Hematoxylin (0.02%). Slides were dewaxed three times with Bond Dewax Solution (Leica Biosystems) at 72 °C, then rehydrated in three steps with graded alcohol and washed with buffer solution (Bond Wash Solution, Leica Biosystems). Antigen retrieval for CB1, CB2 and FAAH was performed by incubating slides with Leica Bond Epitope Retrieval Solution 2 (pH 9.0) for 20 minutes. Primary antibodies diluted in Bond Primary Antibody Diluent (Leica Biosystems) to 1:1000 for CB1 and CB2, and 1:1200 for FAAH were added for 20 minutes, then slides were incubated with post primary polimer for 15 minutes. After washing slides with buffer solution and deionized water, peroxidase activity was blocked by incubation with peroxide block for 3 minutes. After additional washing (buffer and deionized water), mixed DAB refine was added to slides for 10 minutes, followed by deionized washing for three times, and incubation with Hematoxylin for 4 minutes. In between steps, sections were washed with Leica Wash Solution 10x Concentrate (diluted to 1:9 ratio). We used human cerebellum as a positive control.

Images were taken on a Zeiss Axiolmager A2 microscope equipped with an AxioCam ICc 1 camera (Carl Zeiss Ltd., NY, USA) connected to a computer running AxioVision image capture and processing software (version release 4.8.2., Carl Zeiss Ltd.), captured at 200x and 400x magnification.

Statistical analysis

The normality of continuous variables was assessed using the Shapiro-Wilk's *W*-test. As the continuous variables were not normally distributed, non-parametric statistical methods were applied. To compare continuous variables between two groups, the Mann-Whitney *U*-test was used. The Fisher exact and Pearson χ^2 tests were

performed to compare categorical variables between groups. The Spearman rank order correlation was carried out to calculate correlation coefficients. As serum levels of anandamide, sFlt-1 and PlGF showed not-normal distributions, we performed analyses of covariance (ANCOVA) with logarithmically transformed data.

Statistical analyses were carried out using the following software: STATISTICA (version 11; StatSoft, Inc., Tulsa, Oklahoma, USA) and Statistical Package for the Social Sciences (version 22 for Windows; SPSS, Inc., Chicago, Illinois, USA). For all statistical analyses, $p<0.05$ was considered statistically significant.

Data are reported as median (interquartile range) for continuous variables and as number (percentage) for categorical variables.

Results

Circulating anandamide levels in preeclampsia

Serum levels of anandamide were significantly lower in preeclamptic patients than in healthy pregnant women (0.75 (0.44-1.03) ng/ml versus 1.30 (0.76-2.0) ng/ml, $p<0.001$). Preeclamptic patients had significantly higher sFlt-1 levels (12121 (7963-18316) pg/ml versus 2299 (1393-3179) pg/ml, $p<0.001$) and significantly lower PlGF concentrations (71.2 (39.2-86.4) pg/ml versus 256.8 (181.1-421.0) pg/ml, $p<0.001$) as compared to healthy pregnant women. The differences in the above laboratory parameters between the two study groups remained significant even after adjustment for maternal age, gestational age at blood draw, pre-pregnancy BMI and the percentage of primiparas in analyses of covariance (ANCOVA).

In the group of preeclamptic patients, no statistically significant differences were found in serum anandamide levels between patients with mild and severe preeclampsia, between patients with late and early onset of the disease, or between preeclamptic patients with and without intrauterine growth retardation. Serum sFlt-1 concentrations were significantly higher in patients with early onset preeclampsia than in those with late onset disease (14606 (12239-24333) pg/ml versus 7971 (5991-12805) pg/ml, $p<0.001$). In addition, preeclamptic patients with fetal growth restriction (41.1 (28.0-73.2) pg/ml) or early onset of the disease (41.1 (23.8-68.7) pg/ml) had significantly lower PlGF concentrations than those without IUGR (77.3 (48.2-93.2) pg/ml, $p<0.05$) or with late onset disease (80.3 (66.0-99.4) pg/ml, $p<0.001$).

We also investigated whether the clinical characteristics of the study participants were related to their serum anandamide levels by calculating the Spearman rank order correlation coefficients (continuous variables) or by the Mann-Whitney *U*-test (categorical variables). In healthy pregnant women, serum anandamide concentrations showed significant positive correlations with pre-pregnancy BMI ($R=0.33$, $p<0.05$). Accordingly, in the healthy pregnant group, overweight and obese women (BMI$\geq$25 kg/m^2) had significantly higher serum anandamide levels than those with normal weight (1.80 (1.20-2.60) ng/ml versus 1.06 (0.70-1.61) ng/ml, $p<0.05$). There was no other relationship between clinical features of the study subjects and their serum anandamide

levels in either study group. Furthermore, serum anandamide concentrations did not correlate with serum levels of sFlt-1 and PlGF in our healthy pregnant and preeclamptic groups.

CB1, CB2 and FAAH expression and localization in the placenta

According to densitometry analysis, placental expression of CB1 protein was significantly higher in preeclamptic patients than in normotensive, healthy pregnant women (149.3 (105.0-279.7) % versus 98.1 (67.3-131.0) %, p=0.008). Nevertheless, no significant differences were observed in placental CB2 (105.5 (80.9-133.2) % versus 65.8 (45.5-128.4) %, p>0.05) and FAAH (112.2 (94.7-143.8) % versus 91.2 (63.3-128.4) %, p>0.05) protein expressions between the two study groups.

Strong CB1 immunoreactivity was detected in the syncytiotrophoblast layer, as well as in the endothelial cells of blood vessels. Less intense, but positive staining was found in the decidua, capillary smooth muscle cells, stromal fibroblasts and in the amnion. CB1 immunoreactivity in the previously mentioned localizations was observed both in normal and preeclamptic placenta, however, staining was markedly more intense in preeclampsia.

Immunoreactive CB2 was detected both in normal and preeclamptic placenta, but there was no difference in the staining intensity and localization between the two groups. Syncytiotrophoblasts and stromal fibroblasts showed positive reaction for CB2, although staining was less strong compared to CB1. CB2 immunoreactivity was absent in blood vessels, but detectable in the decidua. We found CB2 immunoreactivity in spot-like distribution in the amnion.

FAAH immunoreactivity showed similar localization to CB2, as we found positive reaction in syncytiotrophoblasts and stromal fibroblasts (however, staining intensity was reduced compared to CB1 immunoreactivity). Immunoreactive FAAH was absent in blood vessels, but detectable in the decidua and less markedly in spot-like distribution in the amnion. FAAH immunoreactive staining intensity and localization was similar in normal and preeclamptic placenta.

Conclusions

1. In our study, we observed decreased circulating anandamide levels in women with preeclampsia, which might be a consequence of its sequestration in the placenta by binding to syncytiotrophoblasts overexpressing CB1. In light of the anti-inflammatory and hypotensive effects of endocannabinoids, it is tempting to speculate that decreased circulating anandamide levels might contribute to the development or strengthening of the excessive systemic inflammatory response and high maternal blood pressure. We did not find a significant correlation between serum anandamide concentration and sFlt-1 and PlGF levels, which suggests that the change in circulating anandamide levels and the angiogenic imbalance are independent processes in preeclampsia.

2. CB1 expression measured by Western blotting was markedly stronger in preeclamptic placenta, and these findings were confirmed by strong CB1 immunoreactivity in various placental localizations. CB1 immunoreactivity was markedly stronger in syncytiotrophoblasts, the mesenchymal core, decidua, villous capillary endothelial and smooth muscle cells, as well as in the amnion in preeclamptic samples compared to normal pregnancies. However, we did not find significant difference between preeclamptic and normal placental tissue samples regarding CB2 and FAAH expression and immunoreactivity. Enhanced CB1 expression could lead to abnormal decidualization and impaired trophoblast invasion, thus playing a role in the pathogenesis of preeclampsia. As endocannabinoids can cause vasodilation, higher CB1 immunoreactivity in villous capillary endothelial and smooth muscle cells could be an adaptive response to poor placentation, enhancing the blood supply of the placenta. While the detailed pathogenesis of preeclampsia is still unclear, the endocannabinoid system could play a role in the development of the disease.

List of publications

Publications related to the subject of the thesis

1. **Fügedi G**, Molnár M, Rigó J Jr, Schönléber J, Kovalszky I, Molvarec A. (2014) Increased placental expression of cannabinoid receptor 1 in preeclampsia: an observational study. BMC Pregnancy Childbirth, 14: 395. (IF: 2,190)
2. Molvarec A, **Fügedi G**, Szabó E, Stenczer B, Walentin S, Rigó J Jr. (2015) Decreased circulating anandamide levels in preeclampsia. Hypertens Res, 38: 413-418. (IF: 2,658)

Publications independent of the subject of the thesis

1. Stenczer B, Molvarec A, Szabó G, Szarka A, **Fügedi G**, Szijártó J, Rigó J Jr. (2012) Circulating levels of thrombospondin-1 are decreased in HELLP syndrome. Thromb Res, 129: 470-473. (IF: 3,133)
2. Toldi G, Vásárhelyi B, Biró E, **Fügedi G**, Rigó J Jr, Molvarec A. (2013) B7 Costimulation and Intracellular Indoleamine-2,3-Dioxygenase Expression in Peripheral Blood of Healthy Pregnant and Pre-Eclamptic Women. Am J Reprod Immunol, 69: 264-271. (IF: 2,668)
3. Molvarec A, Gullai N, Stenczer B, **Fügedi G**, Nagy B, Rigó J Jr. (2013) Comparison of placental growth factor and fetal flow Doppler ultrasonography to identify fetal adverse outcomes in women with hypertensive disorders of pregnancy: an observational study. BMC Pregnancy Childbirth, 13: 161. (IF: 2,152)
4. Gullai N, Stenczer B, Molvarec A, **Fügedi G**, Veresh Z, Nagy B, Rigó J Jr. (2013) Evaluation of a rapid and simple placental growth factor test in hypertensive disorders of pregnancy. Hypertens Res, 36: 457-462. (IF: 2,936)

Cumulative impact factor: 15,737.