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ORIGINAL ARTICLE

Decreased circulating anandamide levels in preeclampsia

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The endocannabinoid system has a key role in female reproduction, including implantation, decidualization and placentation. A growing number of studies indicate that placental and peripheral blood anandamide levels correlate closely with both spontaneous miscarriage and ectopic pregnancy. Anandamide has also been implicated in blood pressure regulation. In this study, we aimed to determine circulating anandamide levels in preeclampsia for the first time in the literature. Forty-three preeclamptic patients and 71 healthy pregnant women were involved in this case–control study. Serum anandamide concentrations were determined by high-performance liquid chromatography-mass spectrometry technique. Serum total soluble fms-like tyrosine kinase-1 (sFlt-1) and biologically active placental growth factor (PlGF) levels were measured by electrochemiluminescence immunoassay. For statistical analyses, nonparametric methods were applied. Serum levels of anandamide were significantly lower in preeclamptic patients than in healthy pregnant women (0.75 (0.44–1.03) ng ml^{-1} *vs.* 1.30 (0.76–2.0) ng ml^{-1}, $P<0.001$). Preeclamptic patients had significantly higher sFlt-1 levels (12 121 (7963–18 316) pg ml^{-1} *vs.* 2299 (1393–3179) pg ml^{-1}, $P<0.001$) and significantly lower PlGF concentrations (71.2 (39.2–86.4) pg ml^{-1} *vs.* 256.8 (181.1–421.0) pg ml^{-1}, $P<0.001$) as compared with healthy pregnant women. Serum anandamide concentrations did not correlate with serum levels of sFlt-1 and PlGF in our healthy pregnant and preeclamptic groups. In conclusion, we demonstrated for the first time in the literature that serum anandamide concentrations are decreased in women with preeclampsia. However, the cause and consequence of this observation remain to be determined.
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Keywords: anandamide; angiogenic factor; endocannabinoid; preeclampsia; pregnancy

INTRODUCTION

Preeclampsia is a pregnancy-specific disorder, characterized by hypertension and proteinuria developing after week 20 of gestation in previously normotensive women, with a worldwide incidence of 3–8%.[1] It is among the leading causes of maternal, as well as perinatal morbidity and mortality, even in developed countries. Despite extensive research, the etiology and pathogenesis of preeclampsia are not completely understood. Increasing evidence suggests that an exaggerated maternal systemic inflammatory response to pregnancy with an increase in the ratios of peripheral blood Th1/Th2 and Th17/regulatory T cells, a systemic oxidative stress, as well as an imbalance between circulating angiogenic and anti-angiogenic factors plays a crucial role in the pathogenesis of the disease.[2–16]

Endocannabinoids are endogenous ligands that bind to the similar receptor as delta-9-tetrahydrocannabinol (Δ^9-THC), the most potent psychotropic constituent of marijuana. The most studied endocannabinoid is *N*-arachidonoylethanolamine (also known as anandamide), which is reported to have a central role in female reproduction, particularly in decidualization,[17] oviductal transport,[18] preimplantation embryo development,[19] timely embryo implantation and ensuring receptive uterine environment.[20] Tightly regulated anandamide levels are critical for reproductive success, which are accomplished through on-demand enzyme synthesis by the *N*-acylphosphatidylethanolamine-specific phospholipase D and degradation via fatty acid amide hydrolase (FAAH).[21] Anandamide binds to the cannabinoid receptors type 1 and 2 (CB1 and CB2), which exert their effects through various signaling pathways including adenylyl cyclase inhibiton leading to decreased cyclic adenosine monophosphate levels, activation of mitogen-activated protein kinases and either activation or inhibition of different ionic channels.[22]

Various pathological reproductive conditions are associated with dysregulated anandamide levels. In mice, higher anandamide levels inhibit blastocyst differentiation, oviductal transport, implantation and trophoblast proliferation.[23] Studies have shown that low FAAH and high CB1 expression in human placenta characterize spontaneous miscarriage,[24] whereas both low FAAH and CB1 expressions causing high anandamide levels in fallopian tubes may have a role in ectopic implantation.[25] High maternal peripheral blood endocannabinoid levels modulated by low FAAH activity in lymphocytes can inhibit successful pregnancy after *in vitro* fertilization,[26,27]

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and were recently associated with ectopic pregnancy possibly through tubal dysfunction.[28]

Endocannabinoids have also been implicated in blood pressure regulation.[29] Endocannabinoids can cause vasodilation through CB1, TRPV1 and nitric oxide (NO)-mediated or NO-independent mechanisms.[30] Anandamide exerts its vasorelaxant effect on endothelial cells in various ways, such as by upregulating the expression and activity of the inducible NO synthase (NO-mediated pathway).[31] In the cerebral circulation, CB1 also has vasodilatory effects directly on vascular smooth muscle by inhibiting calcium entry through L-type calcium channels.[32]

While a growing number of studies indicate that placental and peripheral blood anandamide levels correlate closely with both spontaneous miscarriage and ectopic pregnancy, there are no data regarding the association between circulating endocannabinoid levels and preeclampsia. In this study, we aimed to determine circulating anandamide levels in preeclampsia for the first time in the literature.

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.

Preeclampsia was defined by increased blood pressure ($\geqslant$140 mm Hg systolic or $\geqslant$ 90 mm Hg diastolic on $\geqslant$ 2 occasions at least 6 h apart) that occurred after week 20 of gestation in a woman with previously normal blood pressure, accompanied by proteinuria ($\geqslant$ 0.3 g per 24 h or $\geqslant$ 1+on dipstick in the absence of urinary tract infection). Blood pressure returned to normal by postpartum week 12 in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure $\geqslant$ 160 mm Hg systolic or $\geqslant$ 110 mm Hg diastolic, or proteinuria $\geqslant$ 5 g per 24 h (or $\geqslant$ 3+on dipstick). Early onset of preeclampsia was defined as onset of the disease before week 34 of gestation (between completed weeks 20 and 33 of gestation). An infant was considered small-for-gestational-age (SGA) if the birth weight was below the tenth percentile for gestational age and gender, according to a Hungarian birth weight percentile table.[33]

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.

Laboratory methods

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 min. The aliquots of serum were stored at − 80 °C until the measurements.

Serum anandamide concentrations were determined by high-performance liquid chromatography-mass spectrometry technique. Serum samples were supplemented with internal standard solution (100 ng ml^{-1} D_4-anandamide), and analytes were extracted with acetonitrile. The clear supernatant was evaporated in N_2, and re-dissolved samples were analyzed by high-performance liquid chromatography-mass spectrometry 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^{-1} anandamide. High-performance liquid chromatography system consisted of a Jasco X-LC binary pump and autosampler (JASCO Benelux B.V., De Meern, Utrecht, The Netherlands). The quantification was performed using Finnigan TSQ Quantum Discovery triple-quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) with electrospray ionization source operated in positive ion mode. Analytes were separated using reversed-phase liquid chromatography on a Purospher STAR C18 column (Merck KGaA, Darmstadt, Germany) with gradient elution. (Mobile phases were 100 mM ammonium acetate (0.1% formic acid) and methanol at a flow rate of 300 µl min^{-1}.)

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. nos. 05109523 and 05144671, respectively) on a Cobas e 411 analyzer (Roche, Mannheim, Germany).

Statistical analysis

The normality of continuous variables was assessed using the Shapiro–Wilk's *W*-test. As the continuous variables were not normally distributed, nonparametric 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 skewed distributions, we performed analyses of covariance with logarithmically transformed data.

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

In the article, data are reported as median (interquartile range) for continuous variables and as number (percentage) for categorical variables.

RESULTS

Patient characteristics

The clinical characteristics of the study participants are shown in Table 1. There were no statistically significant differences in terms of maternal age, gestational age at blood sampling and the percentage of smokers between the two study groups. The pre-pregnancy body mass index (BMI), the systolic and diastolic blood pressures and the percentage of primiparas were significantly higher, whereas the gestational age at delivery and the fetal birth weight were significantly lower in the preeclamptic group than in the control group. Twelve infants were SGA in the preeclamptic group. Twenty-seven women had severe preeclampsia and 20 patients experienced early onset of the disease.

Laboratory parameters

The laboratory parameters of the study subjects are displayed in Figures 1–3. Serum levels of anandamide were significantly lower in preeclamptic patients than in healthy pregnant women (0.75 (0.44–1.03) ng ml^{-1} *vs.* 1.30 (0.76–2.0) ng ml^{-1}, $P<0.001$; Figure 1). Preeclamptic patients had significantly higher sFlt-1 levels (12 121 (7963–18 316) pg ml^{-1} *vs.* 2299 (1393–3179) pg ml^{-1}, $P<0.001$; Figure 2) and significantly lower PlGF concentrations (71.2 (39.2–86.4) pg ml^{-1} *vs.* 256.8 (181.1–421.0) pg ml^{-1}, $P<0.001$; Figure 3) as compared with 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.

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

Table 1 Clinical characteristics of healthy pregnant women and preeclamptic patients

	Healthy pregnant women (n = *71*)	*Preeclamptic patients* (n = *43*)	*Statistical significance* (P-*value*)
Age (years)	31 (28–34)	30 (28–34)	NS
Pre-pregnancy BMI (kg m^{-2})	22.0 (20.2–25.0)	24.0 (22.0–27.0)	<0.05
Smokers	8 (11.3%)	4 (9.3%)	NS
Primiparas	24 (33.8%)	27 (62.8%)	<0.05
Systolic blood pressure at blood draw (mm Hg)	110 (100–120)	160 (150–170)	<0.001
Diastolic blood pressure at blood draw (mm Hg)	70 (60–80)	101 (100–110)	<0.001
Gestational age at blood draw (weeks)	37 (32–38)	35 (32–37)	NS
Gestational age at delivery (weeks)	39 (39–40)	35 (32–38)	<0.001
Fetal birth weight (g)	3440 (3200–3820)	2260 (1700–3000)	<0.001
Small-for-gestational-age infants	0 (0%)	12 (27.9%)	<0.001

Abbreviations: BMI, body mass index; NS, not significant.
Data are presented as median (interquartile range) for continuous variables and as number (percentage) for categorical variables.

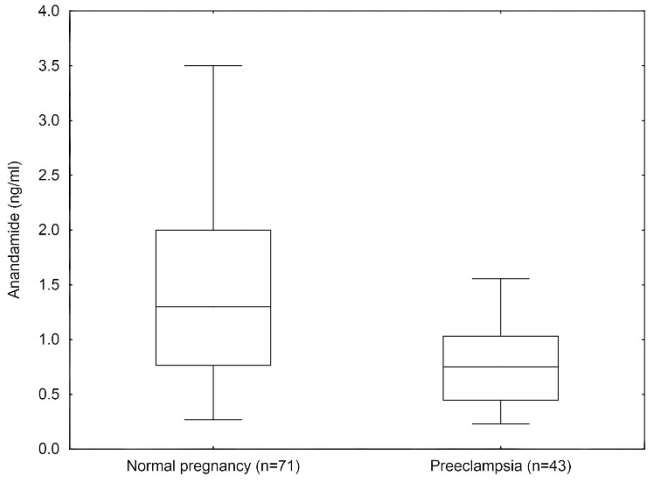

Figure 1 Serum anandamide levels of healthy pregnant women and preeclamptic patients ($P<0.001$). Middle line: median; box: interquartile range (25–75 percentile); whisker: range (excluding outliers).

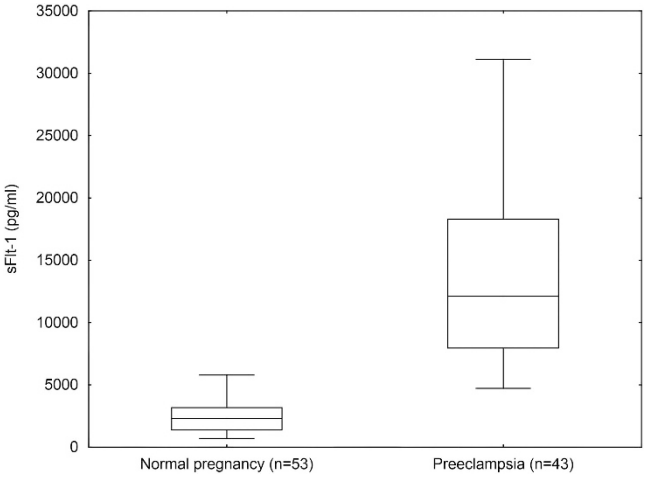

Figure 2 Serum soluble fms-like tyrosine kinase-1 (sFlt-1) levels of healthy pregnant women and preeclamptic patients ($P<0.001$). Middle line: median; box: interquartile range (25–75 percentile); whisker: range (excluding outliers).

early onset of the disease or between preeclamptic patients with and without an small-for-gestational-age infant (data not shown). Serum sFlt-1 concentrations were significantly higher in patients with early onset preeclampsia than in those with late onset disease (14 606 (12 239–24 333) pg ml^{-1} *vs.* 7971 (5991–12 805) pg ml^{-1}, $P<0.001$). In addition, preeclamptic patients with an SGA infant (41.1 (28.0–73.2) pg ml^{-1}) or early onset of the disease (41.1 (23.8–68.7) pg ml^{-1}) had significantly lower PlGF concentrations than those without an SGA infant (77.3 (48.2–93.2) pg ml^{-1}, $P<0.05$) or with late onset disease (80.3 (66.0–99.4) pg ml^{-1}, $P<0.001$).

We also investigated whether the clinical characteristics of the study participants presented in Table 1 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 $\geqslant 25$ kg m^{-2}) had significantly higher serum anandamide levels than those with normal weight (1.80 (1.20–2.60) ng ml^{-1} *vs.* 1.06 (0.70–1.61) ng ml^{-1}, $P<0.05$). There was no other relationship between clinical features of the study subjects and their serum anandamide levels in

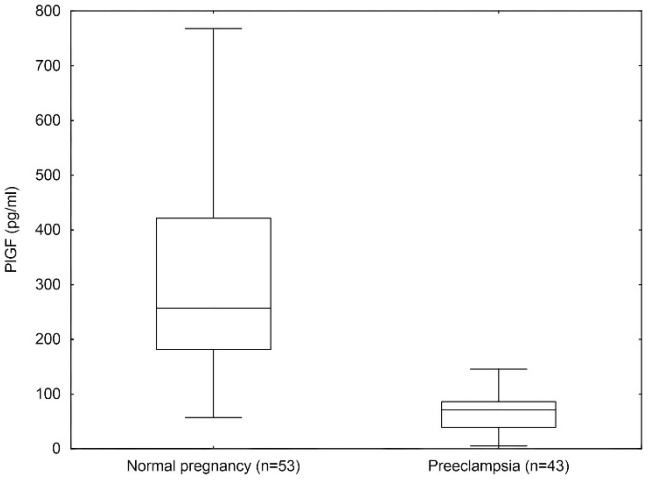

Figure 3 Serum placental growth factor (PlGF) levels of healthy pregnant women and preeclamptic patients ($P<0.001$). Middle line: median; box: interquartile range (25–75 percentile); whisker: range (excluding outliers).

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.

DISCUSSION

In this study, we measured circulating levels of anandamide along with those of sFlt-1 and PlGF in normal pregnancy and preeclampsia. According to our findings, serum anandamide levels are decreased in women with preeclampsia. However, circulating levels of anandamide were not related to those of angiogenic factors in preeclampsia.

In the healthy pregnant group, we found a significant positive correlation between pre-pregnancy BMI and anandamide levels. In accordance, anandamide concentrations were significantly higher among overweight and obese healthy pregnant women. This observation is consistent with earlier findings, which demonstrated a link between overactivated endocannabinoid system and obesity. Activation of the central endocannabinoid system increases food intake and promotes weight gain. In addition, a strong negative correlation was found between FAAH expression in human adipose tissue and circulating anandamide levels.[34,35] CB1 agonists increasing lipoprotein lipase activity and ultimately enhancing lipogenesis in mouse adipocytes *in vitro* may explain how anandamide redounds to obesity with a peripheral mechanism.[36] However, we did not find such an association in the preeclamptic group. In fact, we even observed lower anandamide levels despite of higher pre-pregnancy BMI. This indicates that other processes may account for changes in circulating anandamide levels in preeclampsia.

In our previous study, we determined CB1, CB2 and FAAH expressions and localization in placental samples from healthy and preeclamptic women.[37] According to our findings, 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, including syncytiotrophoblasts. Therefore, decreased serum concentration of anandamide in preeclampsia might be a consequence of its sequestration in the placenta by binding to syncytiotrophoblasts overexpressing CB1. Nevertheless, Aban *et al.*[38] detected similar CB1 protein expression in normal and preeclamptic placental tissues.

Earlier studies have shown that leptin stimulates FAAH activity in human T-lymphocytes[39] and human lymphoma cells.[40] Furthermore, interleukin (IL)-6 also increases FAAH activity in human lymphocytes.[41] Both leptin and IL-6 serum levels are elevated in preeclampsia.[7,42] Thus, increased lymphocyte FAAH expression and activity mediated by leptin or IL-6 might be another candidate, which is responsible for decreased circulating anandamide levels in preeclampsia. Further studies are needed to clarify this issue with measurement of lymphocyte FAAH expression in the peripheral blood of preeclamptic and healthy pregnant women.

Heat shock protein 70 and albumin can act as cytosolic anandamide-binding proteins.[43] We have recently reported that Hsp70 serum levels are elevated in preeclampsia.[44–46] It is also possible that Hsp70 as a chaperone binds anandamide in the peripheral circulation leading to its decreased serum levels. Moreover, by binding to albumin, anandamide could be excreted through the kidneys with loss of albumin in the urine. Nevertheless, we did not find any relationship between circulating anandamide levels and the degree of proteinuria in our preeclamptic pregnant women.

Recent findings indicate a central role of circulating angiogenic factors and their antagonists in the pathogenesis of preeclampsia.[3] In this study, we also measured serum sFlt-1 and PlGF concentrations in normal pregnancy and preeclampsia by electrochemiluminescence immunoassay. However, increased sFlt-1 and decreased PlGF levels were not related to serum anandamide concentrations in women with preeclampsia, suggesting that alterations in circulating levels of angiogenic factors and anandamide are independent processes in preeclampsia.

Both exogenous and endogenous cannabinoids have been reported to exert anti-inflammatory effects. Anandamide attenuates Th17-mediated inflammation through *in vivo* increasing IL-10 production, which induces different microRNAs targeting pro-inflammatory cytokines and leading to IL-17 and interferon-γ reduction.[47] Endocannabinoids also mediate cytokine shift from Th1 to Th2 cytokines by suppression of cell activation, inhibition of pro-inflammatory cytokine production and nuclear factor-κB-dependent apoptosis.[48] Both anandamide and exogen Δ^9-THC increase regulatory T-cell functions through CB1 and CB2, resulting in significant suppression of inflammatory cytokines and chemokines.[49] Therefore, it is tempting to speculate that decreased circulating anandamide levels might contribute to the development of the excessive systemic inflammatory response and the increase in the ratios of peripheral blood Th1/Th2 and Th17/regulatory T cells characteristics of the maternal syndrome of the disease.

Chronic activation of the endocannabinoid system results in complex cardiovascular effects, primarily in bradycardia, decrease of blood pressure and myocardial contractility, mediated by CB1 via stress signaling and cell death leading to decreased contractility.[50] Underlying mechanisms involve modulation of autonomic outflow through presynaptic autonomic nerve terminals and the central nervous system, as well as direct myocardial effects.[30] Vasodilatory effects may involve CB1 and TRPV1 receptor- and NO-mediated or NO-independent pathways. Thus, it is also possible that decreased serum anandamide concentration might have a role in blood pressure elevation in preeclampsia. Although we did not find a significant correlation between anandamide levels and blood pressure values in our preeclamptic group, even sFlt-1 and PlGF levels were not related to blood pressure values or the severity of the disease, indicating that regulation of blood pressure is more complex in this pregnancy-specific disorder. Interestingly, in a recent study, increased angiotensin II contraction of the uterine artery at early gestation in a transgenic rat model of preeclampsia was reduced by inhibition of anandamide hydrolysis, suggesting that renin–angiotensin system-endocannabinoid system interactions may contribute to the enhanced vascular reactivity in early stages of hypertensive pregnancy.[51]

In our study, the similar serum anandamide levels of preeclamptic patients regardless of the severity, the time of onset of the disease or the presence of an small-for-gestational-age infant might be explained by the complex etiology of preeclampsia. Several genetic, behavioral and environmental factors need to interact to produce the complete picture of this pregnancy-specific disorder. We reported various genetic and soluble factors that were associated with the severity or complications of preeclampsia, including HELLP syndrome and fetal growth restriction.[45,52–54] Nevertheless, it is also possible that the relatively small sample size of the preeclamptic group prevented to detect an effect in the subgroup analyses.

In conclusion, we demonstrated for the first time in the literature that serum anandamide concentrations are decreased in women with preeclampsia. However, the cause and consequence of this observation remain to be determined.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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