

Elevated Anandamide and Related *N*-Acylethanolamine Levels Occur in the Peripheral Blood of Women With Ectopic Pregnancy and Are Mirrored by Changes in Peripheral Fatty Acid Amide Hydrolase Activity

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Background: Studies from knockout mice suggest that perturbations in oviductal endocannabinoid levels, endocannabinoid receptors, or endocannabinoid degrading enzyme [fatty acid amide hydrolase (FAAH)] expression result in infertility secondary to physical trapping of embryos. Similar observations have been made in ectopic pregnant women together with a suggestion that the endocannabinoid receptor gene polymorphism 1359G/A (rs1049353) is associated with ectopic pregnancy. These observations led to the hypothesis that ectopic pregnancy is associated with a perturbation in levels of endocannabinoids and FAAH activity and that such changes are associated with impaired tubal function.

Aims: The objective of the study was to quantify the plasma levels of endocannabinoids (anandamide, oleoylethanolamide, and palmitoylethanolamide) and evaluate blood endocannabinoid metabolizing enzyme activities FAAH and *N*-acyl-phosphatidyl-ethanolamine phospholipase D (NAPE-PLD) in ectopic pregnancy and normal pregnant controls and relate that to β -human chorionic gonadotropin (β -hCG) levels. Additionally, we wanted to examine the effect of endocannabinoids on cilia beat frequency in Fallopian tube epithelial cells ex vivo.

Participants and Methods: Whole blood collected from ectopic and normal pregnancies was used for quantification of plasma endocannabinoid levels by ultra-HPLC-tandem mass spectrometry of FAAH and NAPE-PLD enzyme activities by radiometric assays, and β -hCG by immunoassay. Fallopian tube epithelial cells from healthy volunteers were treated with endocannabinoids and cilia beat frequency analyzed using a high-speed digital camera and CiliaFA software.

Results: FAAH activity ($P < .05$) but not NAPE-PLD activity was significantly reduced in ectopic pregnancies. All 3 endocannabinoids levels were significantly higher ($P < .05$) in ectopic pregnancy. There was no correlation between endocannabinoids, enzyme activity, and β -hCG levels. Oleoylethanolamide ($P < .05$), but not methanandamide or palmitoylethanolamide, significantly decreased cilia beat frequency in Fallopian tube epithelial cells.

Conclusion: Elevated endocannabinoid levels and reduced FAAH activity are associated with ectopic pregnancy and may modulate tubal function, suggesting dysfunctional endocannabinoid action in ectopic implantation. Oleoylethanolamide may play a critical role in embryo-tubal transport. (*J Clin Endocrinol Metab* 98: 1226–1234, 2013)

Human reproduction is notoriously inefficient with 40%–50% of all conceptions failing to progress beyond 20 weeks of gestation (1, 2). Although 75% of these unsuccessful pregnancies represent failure of implantation (1), a significant proportion are ectopic pregnancies. The current management of ectopic pregnancy presents both a challenge and a financial burden on health care providers (3). Although there have been improvements in the care of women with ectopic pregnancy in developed countries, the associated morbidity and mortality remain significantly higher in the less resourced countries (4, 5). A limited understanding of the mechanisms involved in tubal implantation may explain our failure to develop preventive therapies and the fact that a significant number of maternal deaths in the first trimester are due to complications of ectopic pregnancy further strengthens the argument for more research in this area (6). In a study of 803 women with ectopic pregnancy, smoking, pelvic inflammatory disease (PID), a history of previous ectopic pregnancy, and a previous tubal surgery were associated with an increased risk of ectopic pregnancy. However, more than 50% of cases had no history of PID or prior tubal surgery, suggesting that other factors may be involved (7).

Cannabinoids (exo- and endocannabinoids) are associated with adverse reproductive events including defective oviductal transport and inhibition of cytotrophoblast cell proliferation (8, 9). Endocannabinoids are unsaturated bioactive fatty acid amides that are ligands for the G protein-coupled cannabinoid receptor types 1 (CB₁) and CB₂. Levels of anandamide (AEA; *N*-arachidonylethanolamine), the prototypical endocannabinoid, are mainly regulated by its synthesizing and degrading enzymes, *N*-acylphosphatidylethanolamine-specific phospholipase-D (NAPE-PLD), and fatty acid amide hydrolase (FAAH), respectively. These enzymes create a conducive internal milieu permitting early pregnancy success (10). Endocannabinoids, their receptors, and metabolizing enzymes are collectively known as the endocannabinoid system (ECS).

Knockout mice studies have demonstrated a clear role for the ECS in modulating oviductal transport; reduced expression of CB₁ or FAAH and high AEA levels in the oviducts of mice was associated with physical trapping of a significant number of embryos compared with their wild-type counterparts (11).

N-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA) are compounds related to AEA, and although they exhibit little or no activity at the cannabinoid

receptors, they are thought to be entourage compounds that modulate the effects of AEA at these receptors by inhibiting AEA degradation, thereby indirectly prolonging its biological effect (12–15). Plasma and tissue levels of OEA and PEA also fluctuate throughout pregnancy, suggesting a possible role in pregnancy maintenance and/or success (16). There is also evidence that these compounds activate the so-called atypical cannabinoid receptors, namely the orphan G protein-coupled receptors (GPRs), GPR55 and GPR119 (17, 18). As with AEA, synthesis and degradation of OEA and PEA are also modulated by NAPE-PLD and FAAH, respectively (19, 20).

With regard to the role of the ECS in ectopic pregnancy, Horne et al (21) demonstrated that CB₁ mRNA levels are attenuated in the Fallopian tubes of women with this complication, when compared with controls, and that the CB₁ gene polymorphism 1359G/A (rs1049353) may be associated with this condition. Additionally, we have recently shown that Fallopian tubes from women with ectopic pregnancy have reduced expression of CB₁ and FAAH and elevated endocannabinoid levels consistent with the knockout mouse data, in which these anomalies in the ECS were associated with physical trapping of embryos in their oviducts (11, 22). Although these findings may indicate potential pharmacological targets for intervention strategies in women at risk of ectopic pregnancy, whether a similar perturbation in endocannabinoid levels and their metabolizing enzymes occur in peripheral blood from these women or whether such changes (if any) are associated with altered tubal function remains unknown. Parallel observations of peripheral plasma levels may therefore act as surrogates for tubal levels and thus identify women at risk of ectopic pregnancy. This led us to hypothesize the following: 1) plasma endocannabinoid levels will be elevated in women with ectopic pregnancy, and 2) this elevation will be regulated by the activities of endocannabinoid modulating enzymes in white blood cells. The aims of the studies presented here were therefore to: 1) quantify the endocannabinoid levels in the peripheral blood from women with ectopic pregnancy and to compare them with those of normal pregnant controls, 2) quantify blood cell FAAH and NAPE-PLD enzyme activities from these samples, and 3) investigate the effects of endocannabinoids on Fallopian tube cilia beat frequency (CBF), which is a process vital to normal embryo-tubal transport (23, 24). Data obtained from these experiments may improve our understanding of the mechanisms un-

derlying tubal implantation and arguably open new horizons for drug therapy targeting the ECS.

Participants and Methods

Ethics statement

Ethical approval was obtained from the Leicestershire and Rutland Local Research Ethics Committee, and all research procedures were conducted according to the principles expressed in the Declaration of Helsinki. All participants signed informed consent forms prior to inclusion into the study.

Participants

The study group comprised women attending as emergencies with a diagnosis of ectopic pregnancy, whereas controls were women with normal pregnancy attending for the termination of pregnancy. All volunteers were recruited from the Leicester Royal Infirmary and Leicester General Hospitals, which are part of the University Hospitals of Leicester National Health Service Trust. Gestation was confirmed in controls using an ultrasound scan-measured crown-rump length, whereas the last menstrual period was used for the women with ectopic pregnancy because it was not possible to estimate gestation using measured crown-rump length in this cohort. For the ciliated epithelial cells studies, normal tubal biopsies were obtained from nonpregnant volunteers attending surgery for total abdominal hysterectomy and bilateral salpingoophorectomy for benign gynecological disease, such as fibroids or dysfunctional uterine bleeding, who were in the luteal phase of the menstrual cycle. The exclusion criteria applied to both the study and control groups included use of any prescription, hormonal or recreational drugs, a history of or current PID, or chronic medical conditions for which they were being treated (eg, chronic airway disease or diabetes mellitus).

Sample collection

Plasma

Venous blood was obtained from the antecubital vein after the application of an appropriate tourniquet. Plasma was isolated from 4 mL of whole blood collected into EDTA monovettes (Sarstedt, Leicester, United Kingdom). For endocannabinoid quantification, samples were centrifuged within 60 minutes of collection at $1200 \times g$ for 30 minutes at 4°C and the plasma transferred immediately into a 7-mL Kimble scintillation vial (Kinesis, St Neots, Cambs, United Kingdom) and, after solid-phase extraction, stored as a dried extract at -80°C (25, 26).

Whole blood

For enzyme activity studies, venous blood (2.7 mL) was collected in plain monovettes (Sarstedt), which were pre-filled with acid citrate dextrose anticoagulant and stored at -80°C . Frozen samples were sent blind to the biochemistry laboratory of the University of Teramo for enzyme activity assays. For analysis, thawed samples were centrifuged at $800 \times g$ for 5 minutes to remove aggregates, and the supernatant was removed and centrifuged again at 4°C and $10\,000 \times g$ for 25 minutes. The final pellet, containing the membrane fractions from all blood cells, was used for enzyme activity assays. All samples were obtained from volunteers within 12 hours of presenting at the hospital and were kept on ice continuously during processing. In women with

ectopic pregnancy, blood samples (including plasma) were collected prior to induction of anesthesia.

Fallopian tubes

Tissue samples were collected immediately after total abdominal hysterectomy and bilateral salpingoophorectomy, washed with sterile PBS and transported to the laboratory in medium 199, supplemented with 25 mM hydroxyethyl piperazine ethane sulfonic acid, Earle's salt, and 100 mM L-glutamine (pH 7.3), 50 $\mu\text{g}/\text{mL}$ streptomycin, and 50 $\mu\text{g}/\text{mL}$ penicillin (Life Technologies, Paisley, United Kingdom). The tubes were used for CBF studies within 2 hours of collection.

Extraction and quantification of endocannabinoids from plasma

A solid-phase method for the extraction and measurement of the endocannabinoids from plasma was as described by Marczylo and colleagues (25, 26) and was used for analysis. Briefly, 2.5 pmol/mL AEA-d8, 2.5 pmol/mL OEA-d2, and 5 pmol/mL PEA-d4 internal standards were added to plasma (0.5 mL) and then diluted to 1 mL with deionized water. Samples were thoroughly mixed on a vortexer for 10 seconds and loaded onto preconditioned Oasis HLB 1-mL cartridges (Waters Ltd, Elstree, United Kingdom). The cartridges were then washed and the endocannabinoids eluted in acetonitrile, as previously described (25, 26). For each volunteer, 2×0.5 mL aliquots of plasma were processed and each extract was analyzed in triplicate. The quantifications of the endocannabinoids and their deuterated equivalents were performed on an ultra-HPLC-tandem mass spectrometry (UHPLC-MS/MS) system, comprising an Acquity ultra-HPLC system in line with a Quattro Premier tandem mass spectrometer (Waters Corp, Milford, Massachusetts). The details of the UHPLC-MS/MS gradient conditions and transitions used have been published previously (25, 26).

Measurement of FAAH and NAPE-PLD activity in peripheral blood cells

NAPE-PLD activity

This was measured from the synthesis of AEA by NAPE-PLD in blood cell membranes as described by Fezza et al (27). Briefly, samples were pooled from the original cohort to obtain a yield of 150 μg membrane protein/test (equivalent to 2–3 individual samples) and incubated in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 30 minutes with 100 μM [^3H]-NArPE (20.8 Ci/mmol) as substrate. The amount of [^3H]-AEA formed was assessed using HPLC with a radiochemical detector as described previously (27).

FAAH activity

This was measured from the hydrolysis of 10 μM [^3H]-AEA (60 Ci/mmol) by FAAH assayed in blood cell membrane samples (50 μg membrane protein/test) by measuring the release of [^3H]-ethanolamine (NEN DuPont de Nemours, Wilmington, Delaware) by means of liquid scintillation counting as described by Gattinoni et al (28). Data for both NAPE-PLD and FAAH activity were expressed as picomoles of product formed per minute per milligram of protein.

Quantification of serum β -human chorionic gonadotropin (β -hCG)

Serum β -hCG was measured by the Biochemistry Department of the University Hospitals of Leicester National Health Service Trust using the ADVIA Centaur Immunoassay System (Bayer HealthCare LLC, Diagnostics Division, Tarrytown, New York) and a total β -hCG 2-sided sandwich immunoassay (Bayer HealthCare LLC, Diagnostics Division) with a detection range between 2 and 1000 mIU/mL. If a level of greater than 1000 mIU/mL was found, a dilution step was performed and the assay repeated, allowing up to 100 000 mIU/mL to be detected (29).

Measurement of CBF

The Fallopian tube was cut open along its whole length using fine scissors and cilia beat recorded and analyzed as described by Hirst and colleagues (30, 31). Briefly, epithelial cells were obtained by brushing the tubal lumen and cells were resuspended in 1 mL of medium 199 (Life Technologies). The sample was then suspended in a chamber created by the separation of a coverslip from a glass slide by 2 adjacent coverslips and placed on a heated stage (37°C) of a Leitz Diaplan microscope mounted on an antivibration table (Wentworth Laboratories Ltd, Sandy, United Kingdom). Cilia beats were observed by using a $\times 100$ Plan apochromat lens and recorded using a digital high-speed video camera (X4 MotionPro camera; IDT, Tallahassee, Florida) at a frame rate of 500 frames per second. Video sequences were recorded and played back at reduced frame rates or frame by frame. Groups of beating cilia (viewed in sideways profile) were identified, and the number of frames required to complete 10 beat cycles was recorded. This was converted to CBF by a simple calculation [CBF = (500/number frames for 10 beats) \times 10] (30, 31) using the CiliaFA software version 1 (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). CBF was recorded from the same epithelial strip before and at various time points (1, 5, 10, 15, and 30 minutes) after treatment with 10 μ M methanandamide, OEA, PEA, or vehicle control (dimethyl sulfoxide). The CiliaFA software is freely available (32).

Statistical analysis

Power analysis of published data with 2-sided $\alpha = .05$ and power of 80% indicated that the minimum number of subjects required in each group that would detect a difference in AEA levels of 20% to be observed was 6 (33). Data are presented as mean \pm SEM (or SD) and comparison between the groups performed using unpaired Student's *t* test, or where appropriate, a 1-way ANOVA followed by an appropriate post hoc analysis, whereas the Pearson's correlation test was used to evaluate the relationships between enzyme activity, endocannabinoid levels, and β -hCG. A probability value of $P < .05$ was accepted as being statistically significant (GraphPad Software, San Diego California; www.graphpad.com).

Results

Participant characteristics

A total of 82 women were recruited for the study. Thirty-eight of these had a normal pregnancy (control group) and 38 had an ectopic pregnancy (study group); these 76 formed the cohort used for the biochemistry studies. The other 6 were

nonpregnant women in the luteal phase of the menstrual cycle who provided the tubal epithelial cells for the ex vivo CBF studies. The mean \pm SD ages (years) of the women studied were 28.0 ± 6.4 for control and 28.6 ± 5.4 for the study groups ($P = .75$, Student's *t* test). Similarly, there were no differences (Student's *t* test) in mean \pm SD body mass index (BMI) and gestational age (BMI: 25.13 ± 4.62 kg/m² vs 26.33 ± 4.81 kg/m², $P = .39$; gestational age: 51.57 ± 8.43 d vs 48.09 ± 6.18 d, $P = .12$) for the control and study groups, respectively. For the measurement of FAAH and NAPE-PLD enzyme activities, 12 samples from the study and 14 from the control groups were used because the collection of these specimens commenced later in the study. Participant characteristics between the 2 groups for the enzyme measurements were similar with no observed differences in age, BMI, or gestation ($P > .05$, Student's *t* test). The mean \pm SD age and BMI of the nonpregnant volunteers were 47.67 ± 3.50 y and 27.85 ± 3.59 kg/m², respectively. A minimum of 3 tubes were used for each endocannabinoid evaluated.

Plasma endocannabinoid levels

The plasma endocannabinoid levels in the study and control groups are shown in Figure 1. AEA levels were significantly higher ($P = .03$) in the study group than in the controls; the mean \pm SEM levels were 0.78 ± 0.04 nM and 0.63 ± 0.04 nM, respectively. Similarly, OEA and PEA levels were significantly higher in the study compared with the control group; 5.20 ± 0.26 nM and 3.70 ± 0.23 nM, $P < .0001$ for OEA and 13.54 ± 1.36 nM and 8.29 ± 1.05 nM, $P = .003$ for PEA. Three women in the study and 5 in the control groups were smokers, and all smoked less than 5 cigarettes per day.

Endocannabinoid modulating enzyme activity in peripheral blood

FAAH activity

Peripheral blood cell membranes from women with ectopic pregnancy showed significantly lower FAAH activity compared with that from normal pregnant controls ($P = .046$, Student's *t* test). The mean \pm SEM FAAH activity expressed as picomoles per minute per milligram protein was: 163.20 ± 9.02 in normal pregnancy ($n = 12$) and 132.00 ± 11.91 in ectopic pregnancy ($n = 10$) (Figure 2). Two samples from each group were excluded from the analysis because their FAAH activities were below the limit of detection.

NAPE-PLD activity

NAPE-PLD activities in peripheral blood cell membranes were not significantly different between the 2 groups. The mean \pm SEM NAPE-PLD activity expressed as picomoles per minute per milligram protein was 50.33 ± 2.78 in normal pregnancy ($n = 6^*$), and $42.21 \pm$

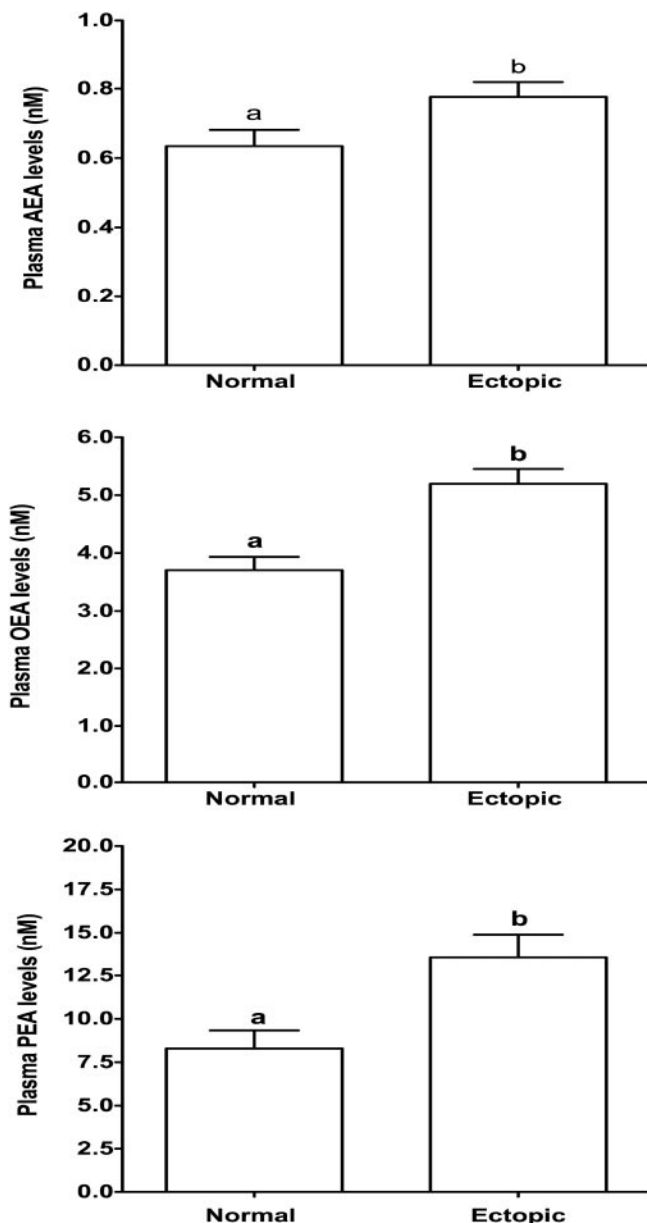


Figure 1. Plasma endocannabinoid levels in ectopic pregnancy. The plasma levels of the endocannabinoids AEA (upper panel), OEA (middle panel), and PEA (lower panel) in ectopic pregnancy ($n = 38$) measured by UHPLC-MS/MS were significantly higher than those from normal pregnant controls ($n = 38$). Endocannabinoid levels are presented as mean \pm SEM, and those with b are significantly different from those with a ($P < .05$), with comparisons being made using Student's t test.

3.25 in ectopic pregnancy ($n = 7^*$) ($P = .10$, Student's t test) (Figure 2). The asterisk indicates that samples were pooled as described in *Materials and Methods*.

Relationship between β -hCG, endocannabinoid levels, and enzyme activity

Serum β -hCG relationship with FAAH and NAPE-PLD activity

There was no significant correlation between blood cell membrane FAAH activity and serum β -hCG levels (Pear-

son's $r = 0.145$, $P = .71$, $n = 10$) or between blood cell membrane NAPE-PLD activity and serum β -hCG levels (Pearson's $r = 0.151$, $P = .75$, $n = 7$).

Serum β -hCG relationship with endocannabinoid levels

Similarly, there was no significant correlation between the levels of AEA and β -hCG (Pearson's $r = 0.071$, $P = .67$, $n = 38$), OEA and β -hCG (Pearson's $r = 0.206$, $P = .22$, $n = 38$), or PEA and β -hCG (Pearson's $r = 0.133$, $P = .43$, $n = 38$).

CBF across the Fallopian tube

CBF were not significantly different between the fimbria, ampulla, and isthmus, although there was a tendency toward a higher CBF in the fimbria compared with the other 2 anatomical sites. A minimum of 10 readings was obtained from each anatomical site from each sample. The mean \pm SEM CBF ($n = 3$) were 14.53 ± 0.46 Hz, 12.67 ± 0.79 Hz, and 12.55 ± 1.60 Hz for the fimbria, ampulla, and isthmus, respectively ($P = .20$; 1 way ANOVA with Bonferroni's multiple comparison test).

Effects of endocannabinoids on Fallopian tube cilia beat frequency

Methanandamide

Prior to the addition of methanandamide, the mean (\pm SEM) baseline CBF for pooled samples from the whole length of the tube was 12.40 ± 4.09 Hz. After the addition of $10 \mu\text{M}$ methanandamide to the epithelial cell strips, CBF initially increased and then decreased, ie, at 1 minute: 13.61 ± 4.44 Hz; 5 minutes: 13.33 ± 1.83 Hz; 10 minutes: 11.23 ± 1.04 Hz; 15 minutes: 9.82 ± 0.33 Hz; and 30 minutes: 9.89 ± 0.94 Hz. Although there was a 20% decrease from baseline CBF at 30 minutes, these observations were not statistically significant ($P = .50$, 1 way ANOVA with Dunnett's multiple comparison test, $n = 3$) (Figure 3).

N-oleoylethanolamine

The baseline mean CBF (\pm SEM) prior to the addition of $10 \mu\text{M}$ OEA was 24.62 ± 7.63 Hz. There was a significant reduction in CBF (60%) after 30 min of treatment ($P = .048$, 1 way ANOVA with Dunnett's multiple comparison test, $n = 3$). The mean CBF (\pm SEM) were as follows: 1 minute: 24.14 ± 8.03 Hz; 5 minutes: 20.95 ± 8.20 Hz; 10 minutes: 17.18 ± 6.21 Hz; 15 minutes: 15.07 ± 4.56 Hz; and 30 minutes: 9.67 ± 0.49 Hz (Figure 3).

N-palmitoylethanolamine

Prior to the addition of $10 \mu\text{M}$ of PEA, the mean CBF (\pm SEM) was 8.19 ± 0.49 Hz. After PEA addition, the readings obtained were: 1 minute: 7.78 ± 0.68 Hz; 5 min-

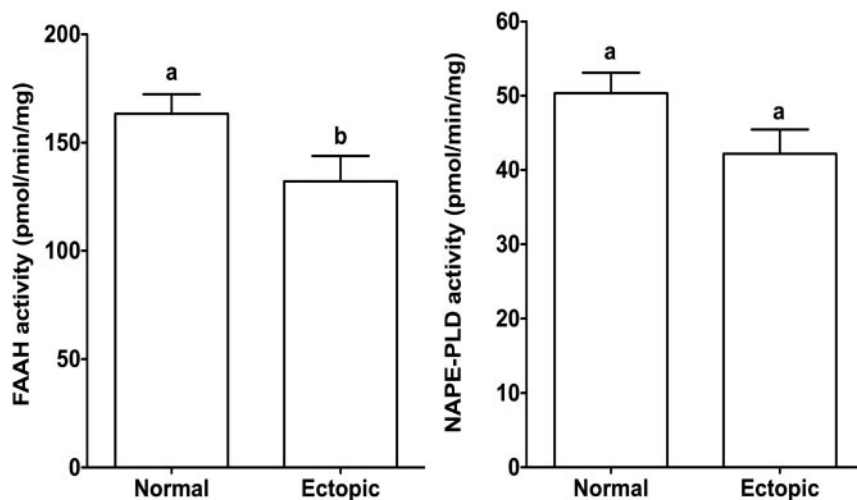


Figure 2. FAAH and NAPE-PLD enzyme activities in peripheral blood cell membranes isolated from pregnant women. FAAH activity (left panel) in ectopic pregnancy samples ($n = 10$) was significantly lower than that of normal pregnant controls ($n = 12$) ($P = .046$; Student's t test). In contrast, NAPE-PLD activity (right panel) was not significantly different between the study ($n = 7^*$) and control ($n = 6^*$) groups. FAAH and NAPE-PLD activities, expressed as picomoles per minute per milligram of protein, are presented as mean \pm SEM, and those with b being significantly different ($P < .05$) from those with a, with comparisons made using a Student's t test. In both experiments, 2 samples were excluded from each group because the FAAH and NAPE-PLD activities in these samples were undetectable. *, Samples were pooled to obtain at least 150 μ g membrane protein per test, as described in *Materials and Methods*.

utes: 7.72 ± 0.20 Hz; 10 minutes: 7.88 ± 0.04 Hz; 15 minutes: 8.07 ± 0.81 Hz; and 30 minutes: 7.76 ± 0.83 Hz ($n = 3$). There was no change in CBF during the course of these experiments compared with baseline observations ($P = .99$, 1 way ANOVA with Dunnett's multiple comparison test, $n = 3$) (Figure 3). For all experiments, there were no differences between the baseline CBF and those from the vehicle control.

Discussion

To our knowledge, this is the first study to quantify 3 different endocannabinoids in the plasma of women with ectopic pregnancy. The key findings were the higher plasma endocannabinoid levels and lower FAAH activities in women with ectopic pregnancy when compared with normal pregnant controls. These findings are particularly interesting, given that animal studies suggest that a high AEA level is associated with poor blastocyst development, arrest of embryos in the oviduct, and implantation failure (11, 34, 35). Indeed, these observations mirror our previous findings of higher endocannabinoid levels in Fallopian tubes obtained from women with ectopic pregnancy compared with nonpregnant luteal phase controls (22) and thus suggests a possible role for these compounds in early pregnancy complications.

The pathways involved in the production and degradation of endocannabinoids are complex, but it is gener-

ally accepted that FAAH and NAPE-PLD are the key degrading and synthesizing enzymes respectively, hence are considered as gatekeepers of endocannabinoid levels (36). Their separate activities and expressions determine local concentrations of these ligands. Indeed, their levels are tightly regulated in early pregnancy such that an appropriate tone is achieved, permitting early pregnancy success (10). Some evidence for this comes from studies by MacCarrone et al (37, 38), in which women with low FAAH activity in lymphocytes and high plasma AEA levels failed to achieve successful pregnancy after in vitro fertilization and embryo transfer procedures. Additionally, in women who became pregnant after natural conception, low lymphocyte FAAH activity, and high plasma AEA levels were associated with miscarriage (38). The data

presented here do suggest that in women with ectopic pregnancy a similar pattern prevails, in which FAAH activity is significantly reduced in peripheral blood cells compared with that of normal pregnant controls. This and the lack of any significant change in NAPE-PLD activity between the 2 groups would favor a higher endocannabinoid level, as has already been demonstrated. These changes in endocannabinoid levels may be modulated by progesterone, a hormone that reduces AEA levels by up-regulating FAAH expression and activity (39). Given that progesterone levels are typically low in ectopic pregnancy compared with normal pregnancy, it may be speculated that this may lead to higher endocannabinoid levels. However, because there was no significant correlation between endocannabinoids, FAAH, or NAPE-PLD activity and serum β -hCG, it could be speculated that this hormone may not have a significant role in endocannabinoid metabolism or if it does, it is poorly defined.

As already noted, this pattern of enzyme activity and substrate (anandamide) level changes has also been demonstrated in women who spontaneously miscarry, and therefore, the specificity of these changes with respect to ectopic pregnancy is questionable and leads one to speculate that these changes are associated with a failing pregnancy rather than any specific clinical entity. It is, however, tempting to speculate from these studies that the outcome of a dysfunctional ECS, eg, ectopic pregnancy or miscarriage, may be dependent on when and where the dysfunction occurs during early pregnancy establishment.

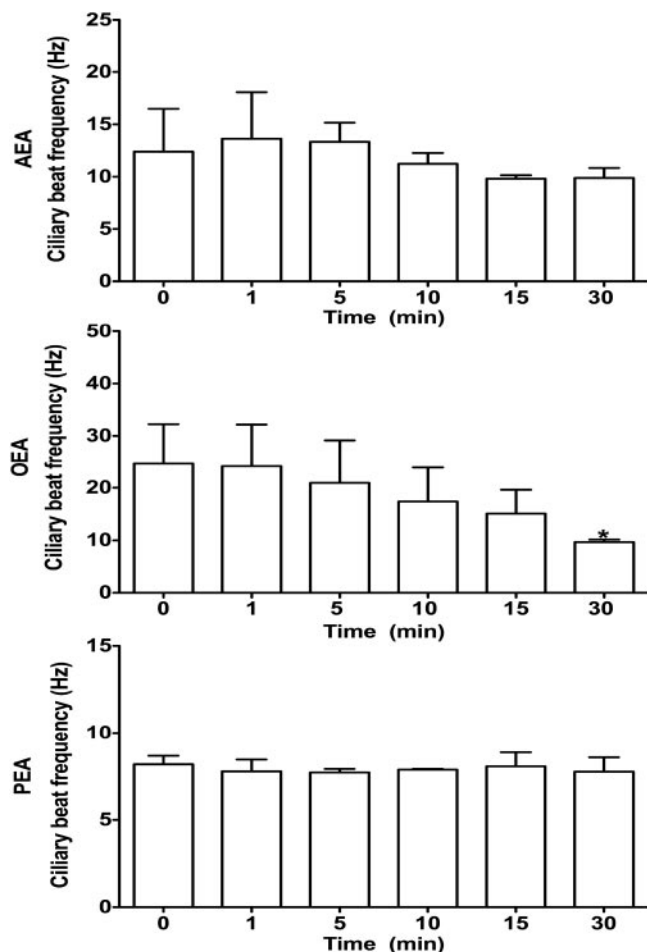


Figure 3. Effects of endocannabinoids on CBF. Graphs show the effects of methanandamide (upper panel), OEA (middle panel), and PEA (lower panel) on CBF ($n = 3$). There were no significant differences in CBF after treatment with $10 \mu\text{M}$ methanandamide ($P = .50$) and PEA ($P = .99$). On the contrary, OEA significantly reduced CBF at 30 minutes ($P = .048$). *, Data are significantly different ($P < .05$) from the baseline CBF. The P values were obtained using a 1-way ANOVA with a Dunnett's multiple comparison test.

The fact that plasma levels of AEA are disturbed in both of these conditions provides a window of opportunity to ascertain the magnitude to which these changes occur in ectopic pregnancy and miscarriage. Such data may provide information on their usefulness as a diagnostic test either by using specific cutoffs of endocannabinoid levels or by combining it with other current markers, such as progesterone or serum β -hCG, to refine assay sensitivity and specificity profiles. What is perhaps more important is the observation of elevated OEA and PEA levels in ectopic pregnancy: endocannabinoids, which have so far not been evaluated in a miscarriage cohort and that are indeed substrate of FAAH (19). FAAH is an enzyme that regulates endocannabinoid levels locally and therefore whether blood FAAH activity truly reflects those in Fallopian tubes remain questionable. However, we have previously shown that FAAH expression is reduced in Fallopian tubes from

women with ectopic pregnancy compared with luteal phase controls, and therefore, it is tempting to suggest that the observations presented here may reflect a global dysfunction of the endocannabinoid system in women with ectopic pregnancy. On a final note, the unprecedented observation that FAAH activity is reduced not only in peripheral lymphocytes but also in whole blood cells of women experiencing fertility problems opens the avenue to a possible diagnostic exploitation of FAAH assays that could be easily performed in whole blood, without the need for time-consuming and application-limiting lymphocyte purifications.

From a disease mechanism perspective, it was necessary to evaluate whether endocannabinoids modulate CBF in Fallopian tube epithelial cells. Smooth muscle contractions and cilia beating are the 2 main processes facilitating normal tubal transport. Derangements in either of these processes may lead to tubal transport arrest and implantation, although the relative contribution of each of the processes to successful embryo-tubal transport is difficult to ascertain and so remains uncertain. However, there is a suggestion that cilia beating alone is capable of propelling oocytes along the oviduct when muscular contractions are inhibited, suggesting that cilia beating may be more important than muscular contractions in normal oviductal transport (23, 24). The results presented here may therefore be helpful in allowing for an objective evaluation of the effects of endocannabinoids on CBF and thus provide crucial information on the etiopathogenesis of ectopic pregnancy.

Reports on the role of endocannabinoids on human Fallopian tube function have not been described, although the role of AEA on smooth muscle contractility has been investigated in the gastrointestinal tract. In a study by Smid et al (40), AEA inhibited cholinergic contractility in human colon, whereas studies with FAAH knockout and wild-type mice suggest that OEA, PEA, and the FAAH inhibitor *N*-arachidonoylserotonin significantly reduced mouse intestinal motility. Unlike smooth muscle contractility studies, those specifically evaluating the effects of endocannabinoids on CBF are rare. Although the expectation was that methanandamide would significantly reduce CBF, the 20% decrease observed in these studies did not reach statistical significance ($P = .50$) (Figure 3 and Supplemental Figure 2). Whether this reduction is clinically significant will remain difficult to ascertain because the numbers involved in these experiments were small and the experiments were mainly observational. Nonetheless, this interesting finding warrants further investigation. PEA did not have any significant effect on CBF after 30 minutes of treatment (Figure 3 and Supplemental Figure 3). What was more important from these functional stud-

ies and a key observation was the finding that OEA significantly inhibited CBF after 30 minutes of treatment (Figure 3 and Supplemental Figure 4). Of note is that higher levels of OEA were demonstrated in both Fallopian tubes (22) and plasma from women with ectopic pregnancy compared with the controls, suggesting that possibly AEA and OEA but not PEA may be involved in modulating ectopic pregnancy, perhaps by inhibiting CBF.

A limitation of this study was the fact that it was observational and that the mean age of the nonpregnant luteal phase samples was higher than would be expected in women with ectopic pregnancy. However, this age difference is largely due to the fact that women attending for benign gynecological surgery have usually completed their families. Nonetheless, the results at the very least suggest that the perturbations demonstrated in the ECS in ectopic pregnant women, leading to higher endocannabinoid levels, may have a functional impact in modulating tubal function. Further studies are, however, required to evaluate the precise mechanisms and pathways involved (if any) of how endocannabinoids may modulate CBF and/or tubal smooth muscle contractility.

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