

Plasma Levels of the Endocannabinoid Anandamide in Women—A Potential Role in Pregnancy Maintenance and Labor?

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Although exposure to exocannabinoids (e.g. marijuana) is associated with adverse pregnancy outcome, little is known about the biochemistry, physiology, and consequences of endocannabinoids in human pregnancy. In these studies, we measured the levels of the endocannabinoid anandamide (*N*-arachidonylethanolamine, AEA) by HPLC-mass spectrometry in 77 pregnant and 25 nonpregnant women. The mean \pm SEM plasma AEA levels in the first, second, and third trimesters were 0.89 ± 0.14 , 0.44 ± 0.12 , and 0.42 ± 0.11 nM, respectively. The levels in the first trimester were significantly higher than those in either the second or third trimester. During labor,

AEA levels were 3.7 times nonlaboring term levels (2.5 ± 0.22 vs. 0.68 ± 0.09 nM, $P < 0.0001$). During the menstrual cycle, levels in the follicular phase were significantly higher than those in the luteal phase (1.68 ± 0.16 vs. 0.87 ± 0.09 nM, $P < 0.005$). Postmenopausal and luteal-phase levels were similar to those in the first trimester. These findings suggest that successful pregnancy implantation and progression requires low levels of AEA. At term, AEA levels dramatically increase during labor and are affected by the duration of labor, suggesting a role for AEA in normal labor. (*J Clin Endocrinol Metab* 89: 5482–5487, 2004)

ENDOGENOUS CANNABINOIDS, endocannabinoids, are unsaturated fatty acid derivatives that act as ligands for the cannabinoid receptors (1). Arachidonylethanolamide or anandamide (AEA) was the first endocannabinoid isolated from brain tissue (2). It is enzymatically released from cell membrane phospholipid precursors in response to depolarizing agents, hormones, and neurotransmitters (3, 4). The levels of AEA are considered to be controlled by its cellular uptake through an AEA transporter (5, 6) and its subsequent enzymatic degradation by a membrane-bound fatty acid amide hydrolase (FAAH) (7–10) and other enzymes (11–13). AEA exerts its effects through interaction with plasma membrane cannabinoid receptors. Two subtypes of cannabinoid receptors, CB1 and CB2, belonging to the superfamily of G protein-coupled receptors (14) have been reported (15–18). They have been localized in different tissue types including the reproductive tract. The CB1 receptor is referred to as the central receptor because it was first localized in the central nervous system (19), whereas the CB2 receptor is referred to as the peripheral receptor because it was first localized in the spleen and other sites (18, 20, 21).

Several epidemiological and observational studies have been published on the adverse effects of exogenous cannabinoids present in marijuana on pregnancy in the human and

animal models. These effects vary from early fetal loss, fetal growth restriction, and premature birth (22–26). By contrast, there are relatively few reports on the role of endocannabinoids in pregnancy. In the mouse, it has been proposed that AEA plays an important role in the local regulation of uterine implantation (27), because AEA was shown to be embryotoxic and arrests embryo development (28). In the implantation site, tissue AEA levels are only 25% of those in the nonimplantation site (29). This is considered to be due to local tissue levels of FAAH, which is expressed at higher levels in the implantation site compared with the nonimplantation site (30). More recent studies have suggested a further potential role for endocannabinoids (e.g. AEA) in myometrium because CB1 receptor is expressed in the human myometrium and *in vitro* stimulation of this receptor by AEA results in relaxation of myometrial strips (31). CB1 receptor and FAAH immunoreactivity have also recently been identified in the human placenta and fetal membranes (32), suggesting that the endocannabinoid system may also be important in these tissues during gestation.

Little information is available on the systemic levels of AEA during human pregnancy. However, during early pregnancy, blood AEA levels are inversely correlated with FAAH levels in peripheral blood mononuclear cells (33), and, since FAAH levels at 8 wk gestation were lower in women who subsequently miscarried compared with those who progressed beyond the first trimester, this suggested that low AEA levels are required for successful pregnancy progression (34). Plasma AEA levels have also been shown to be higher in patients who fail to achieve an ongoing pregnancy after *in vitro* fertilization (IVF) treatment and embryo transfer

Abbreviations: AEA, *N*-Arachidonylethanolamine (anandamide); AEA- d_0 , unlabeled AEA; AEA- d_8 , deuterium-labeled AEA; BMI, body mass index; FAAH, fatty acid amide hydrolase; IVF, *in vitro* fertilization; MS, mass spectrometry.

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compared with those who had successful ongoing pregnancies and were mirrored by lower levels of FAAH in peripheral blood mononuclear cells (33). Whether these differences are influenced by or depend upon the various changes in the sex steroids that modulate implantation and maintain early pregnancy is uncertain. However, the relationship between AEA levels and pregnancy establishment and in any other role is unclear because AEA levels have not been reported beyond the first trimester or indeed during the menstrual cycle. Such studies have been hampered by methodological difficulties in the past, but the tools and techniques used for AEA assays have been and continue to be refined (35).

Our objectives were, firstly, to refine and validate an established method to assay AEA in human plasma and then to use this method to determine, for the first time, plasma levels of AEA in the menstrual cycle and normal pregnancy.

Subjects and Methods

Subjects

All subjects gave signed informed consent to take part in the study, which was approved and conducted according to the guidelines of the institute's research ethics committee. For the purposes of determining the changes in the levels of AEA during pregnancy, we studied cross-sectionally five groups of women in the first, second, and third trimesters; term nonlaboring; and term laboring. To minimize the potential influence of a wide gestational window on AEA levels, we restricted our sampling to 6–11 postmenstrual weeks in the first trimester, 15–27 postmenstrual weeks in the second trimester, and 28–35 postmenstrual weeks in the third trimester. Term was defined as 37–42 completed postmenstrual weeks.

The inclusion criteria for the pregnant women were body mass index (BMI) less than 27 kg/m², accurately dated pregnancies from first-trimester ultrasound scans performed between 6 and 8 postmenstrual weeks, uncomplicated singleton pregnancies, and no coexisting maternal or gestational diseases. None were on any medication or known to have taken recreational drugs. Only women in established labor (defined as cervical dilatation of at least 4 cm and three to four regular uterine contractions every 10 min) were included in the term laboring group.

The nonpregnant women were divided into two groups: pre- and postmenopausal. The inclusion criteria for the premenopausal women included: having had regular menstrual cycles for the last 6 months, having a BMI less than 27 kg/m², not having been on any hormonal contraception or therapy for at least 3 months, and not being on any drugs or suffering from any medical disorders. The samples were collected in two subgroups based on the menstrual cycle (follicular, d 2–7; luteal, d 20–25) assessed from patient-reported last menstrual period date. The inclusion criteria for the postmenopausal women included: a minimum of 12 yr postmenopause, BMI of less than 27 kg/m², not having been on hormone replacement therapy for the preceding 3 months, and not being on any drugs (*e.g.* glucocorticoids, antihypertensives, analgesics, recreational drugs) or suffering from any medical disorders. We studied only nonobese women, defined by a BMI less than 27 kg/m² (in our environment), as we were uncertain about the effect of adiposity on the homeostasis of AEA.

AEA measurements

Plasma AEA was measured by HPLC-mass spectrometry (HPLC-MS) based on an isotope dilution method as reported previously (36). Blood samples (4 ml) were collected in EDTA tubes on ice and added to polypropylene tubes containing 2 ml of Krebs-Tris-EDTA buffer. Samples were centrifuged at 1200 × *g* for 30 min at 22°C to separate plasma from cells. After recovery, the plasma was spiked with 25.1 pmol of deuterium-labeled AEA (AEA-d₈; Cayman Chemicals, Ann Arbor, MI) to estimate the efficiency of the lipid extraction procedure. Protein was precipitated by addition of ice-cold acetone followed by centrifugation at 1000 × *g* for 10 min at 22°C. Lipid extraction was then performed on the supernatants by the addition of methanol:chloroform (1:2) (1:1 vol:

vol). The chloroform layer was recovered, and the samples were dried under a stream of nitrogen before reconstitution in methanol:chloroform (3:1). Duplicate injections of reconstituted samples (10 μl) were used for HPLC-MS analysis.

The HPLC-MS system consisted of a Waters 1525 binary liquid chromatography pump and a Waters 717 plus autosampler fitted with a 100-μl injection loop (Waters MS Technology, Manchester, UK). The HPLC was interfaced to a Quattro Ultima triple quadrupole mass spectrometer via a Z-spray ion source and controlled by MassLynx NT software version 3.5 (Waters MS Technology). Reversed-phase chromatographic separations were performed on a Hypersil octadecylsilane C₁₈ column (4.6 × 100 mm, 3 μm particle size; Phenomenex, Macclesfield, UK). The column temperature was maintained at 30°C using a column temperature controller (Jones Chromatography, Hengoed, UK) and the samples at 4°C in a refrigerated injection system. The mobile phase consisted of water (A) and methanol (B) delivered at 0.5 ml/min using a stepwise increase in methanol, thus: 25% A, 75% B for 2 min; 15% A, 85% B for 3 min; 5% A, 95% B for 20 min; 100% B for 5 min; a column re-equilibration step was then performed by changing the buffer conditions to 25% A, 75% B for 20 min. Under these conditions, the retention times for AEA and AEA-d₈ were 17.12 and 16.98 min, respectively.

Electrospray ionization was carried out in the positive mode using nitrogen as the nebulizing gas. Capillary voltage was set at 3.5 kV, cone voltage 50 V, source temperature 120°C, and desolvation temperature 350°C. MS parameters for unlabeled AEA (AEA-d₀) and AEA-d₈ analysis were established by infusion standards at 1 ng/μl using an infusion pump. Sodium adducts of the molecular ions [M + Na⁺; *m/z* = 370.3 (d₀) and 378.3 (d₈)] were used for quantification in the selected ion recording mode. The ratio of the area under the curve for AEA-d₀ to AEA-d₈ was determined, and quantification of the amount of AEA in plasma samples spiked with the same amount of AEA-d₈ was made directly from a plot of area ratios *vs.* known AEA-d₀ amounts (Cayman Chemicals) injected into the HPLC. Standard curves were derived from the quantification of known and increasing quantities of the labeled AEA. The recovery of the AEA was constant over these ranges of concentrations. This enabled validation of the method of quantification. In addition, before quantification of AEA on the study subjects, we had undertaken repeated (three per volunteer from the same blood sample) measurements from nonpregnant adult volunteers to determine the reliability of recovery. The values obtained from each subject after repeated measurements were very similar and reassured us of the recovery. The standard curves were linear up to 2 pmol AEA-d₀, with a lower limit of detection of 0.03125 pmol AEA-d₀ injected on the column. The intra- and interassay coefficients of variation for the range of detection (0.03–2.1 pmol/column) were 6 and 9%, respectively.

All the blood samples were processed within 2 h of collection as recommended by Giuffrida *et al.* (36). Failure to process the sample within this time period led to degradation of AEA, resulting in a recovery of less than 50% (data not shown).

Statistical analyses

Power analysis of published AEA data (33) with $\alpha = 0.05$ and $\beta = 0.8$ indicated that the minimum number of subjects required in each study group that would allow a significant change in plasma AEA concentrations to be observed was six. Therefore, we ensured that at least six subjects were included in each sample group (Table 1). Data are expressed as mean ± SEM or SD where appropriate for each test group, and comparison between groups was performed using the unpaired Stu-

TABLE 1. Distribution, age, and gestational age of the study groups (data are presented as means with ranges in parentheses)

Study group	No.	Age (yr)	Gestational age (wk)
First trimester	10	30.4 (25–36)	9.1 (6–11)
Second trimester	10	28.1 (20–37)	18.7 (14–26)
Third trimester	10	26.3 (21–34)	31.9 (28–35)
Term (not laboring)	22	30.4 (22–37)	39.3 (37–42)
Term (laboring)	25	29.5 (18–40)	39.5 (37–42)
Menstrual cycle (follicular)	9	31.2 (23–39)	
Menstrual cycle (luteal)	8	34.2 (22–40)	
Postmenopausal	8	57.7 (55–60)	

dent's *t* test with Welch's correction for data with nonequivalent sds; a $P < 0.05$ was considered statistically significant.

Results

Subjects

One hundred two pregnant, menstruating, and postmenopausal women were studied. There were 10 each in the first, second, and third trimesters; 22 in term and nonlaboring groups; and 25 in term and laboring groups. Table 1 shows the details of these women. There were no significant differences in the mean \pm SD ages of the women of reproductive age, but the mean \pm SD of the postmenopausal women was significantly greater than that of the nonpregnant menstruating group (57 ± 2.0 yr, *cf.* 32.7 ± 6.1 ; $P < 0.05$). The mean \pm SD gestational ages of the term nonlaboring and laboring women were similar (39.3 ± 1.3 yr, *cf.* 39.6 ± 1.3 ; $P > 0.05$).

Seventeen of the 25 women in the term laboring group had a spontaneous vaginal delivery, three had a forceps delivery, two had a vacuum extraction, and three had an emergency cesarean section. The mean \pm SD cervical dilatation at the time of sampling was 6.04 ± 2.2 cm (range 4–10 cm), whereas the mean duration of uterine contractions before the blood sample collection was 5.1 ± 2.2 h (range 1.2–12 h). The mean \pm SD interval between sampling and delivery was 5.3 ± 4.3 h (range 0.5–11.8 h). Thirteen women had epidural analgesia in labor before sampling, and the others (12) had only a combination of nitrous oxide and oxygen, otherwise known as “gas and air,” in labor.

AEA levels

The levels of AEA during pregnancy, excluding the laboring women, fell from 0.89 ± 0.14 nM in the first trimester to 0.44 ± 0.12 nM in the second trimester and 0.44 ± 0.11 nM in the third trimester ($P = 0.04$) and thereafter rose to 0.68 ± 0.09 nM in the term nonlaboring group ($P = 0.03$). There was, however, a very dramatic change in the levels in the term laboring women, which rose 3.7 times the values in term nonlaboring women and six times those in the third trimester to 2.5 ± 0.22 nM. The differences between term laboring and the first, second, and third trimesters and nonlaboring levels were all statistically significant ($P < 0.0001$).

In the menstruating women, the levels of AEA in the follicular phase (1.68 ± 0.16 nM) were significantly higher than those in the luteal phase (0.87 ± 0.19 nM) ($P < 0.0053$). The levels in postmenopausal women (0.67 ± 0.01 nM) were significantly lower than those in the follicular phase ($P = 0.0002$) but not significantly lower than those in the luteal phase.

When the levels of AEA in the menstruating and postmenopausal women were compared with those during pregnancy, significant patterns emerged. These are shown in Fig. 1. The postmenopausal (0.67 ± 0.01 nM) and luteal-phase levels (0.77 ± 0.19 nM) were similar to those in the first trimester, higher (but not statistically, $P > 0.05$) than the second and third trimesters and nonlaboring term levels but significantly lower than the levels in the term laboring group ($P < 0.0002$). The follicular-phase levels were significantly higher ($P < 0.001$) than the levels during pregnancy but significantly lower ($P < 0.005$) than those in the laboring group.

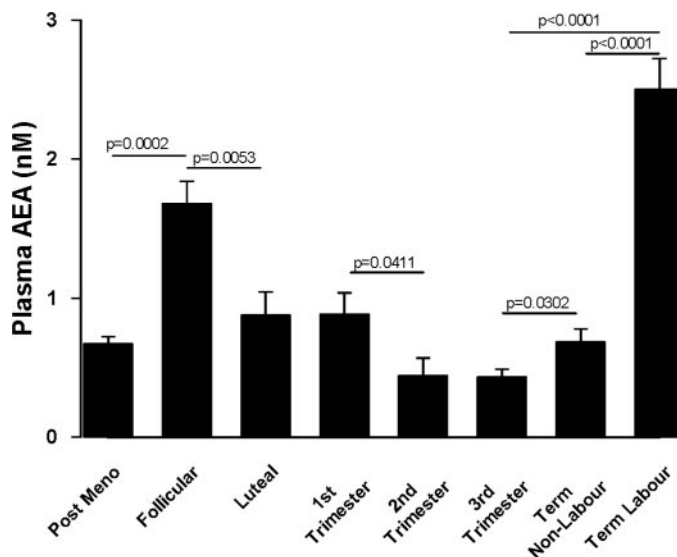


FIG. 1. Plasma AEA levels in postmenopausal women, during the menstrual cycle, pregnancy, and in nonlabor and labor women at term. Data are means \pm SEM; n values are shown in Table 1. Comparisons were performed using the Student's unpaired *t* test with Welch's correction. $P < 0.05$, first trimester *vs.* second trimester and third trimester *vs.* term nonlaboring; $P < 0.0001$, laboring *vs.* term nonlaboring or third trimester; $P < 0.001$ postmenopausal *vs.* follicular phase of the menstrual cycle; $P < 0.001$ follicular *vs.* luteal phase of the menstrual cycle.

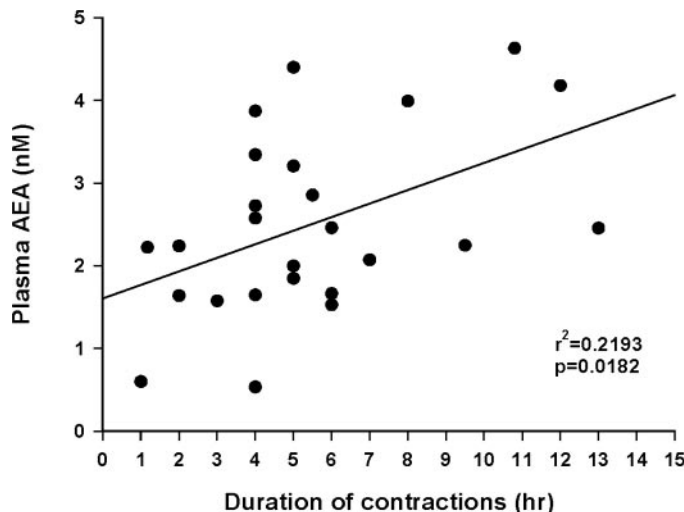


FIG. 2. Relationship between plasma AEA levels and the duration of contractions at the time of sampling.

Figure 2 shows the relationship between plasma AEA levels and the duration of contractions up to the time of sampling. There was a direct linear relationship between AEA levels and this duration ($r^2 = 0.2193$, $P < 0.0182$). The laboring women were further divided into three groups based on the total duration of labor (group I, 0–5 h; group II, 5.1–10 h; and group III, 10.1–15 h). The mean \pm SEM levels of AEA in the three groups were, respectively, 2.1 ± 1.04 , 2.58 ± 0.95 , and 3.8 ± 1.15 nM. Although there was a trend for AEA values to increase with increasing duration of labor, this did not achieve statistical significance ($P > 0.05$). Figure 3 shows an inverse relationship between the levels of AEA at the time of

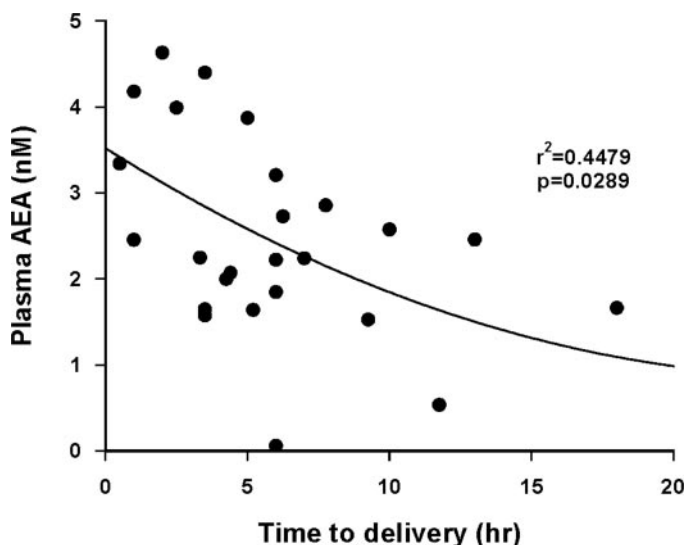


FIG. 3. Relationship between plasma AEA levels and the interval from sampling to delivery.

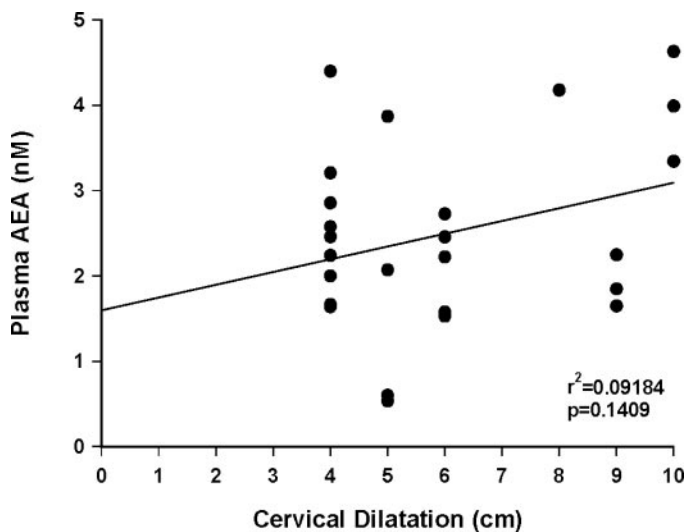


FIG. 4. Relationship between plasma AEA levels and cervical dilatation at the time of sampling.

sampling and the time from sampling to delivery. Figure 4 shows the relationship between the cervical dilatation at the time of sampling and plasma AEA levels ($r^2 = 0.092$, $P = 0.1$). The relationship between plasma AEA and the interval between sampling and delivery is shown in Fig. 3 ($r^2 = 0.4479$, $P = 0.0289$).

The levels in the 13 women who had an epidural analgesia before sampling were similar (2.24 ± 0.32 nM) to those in the 12 that had entonox gas only (2.78 ± 0.30 nM) before sampling and for the rest of their labor. None of the women received opiates.

Discussion

Our results demonstrate remarkable changes in the levels of AEA during pregnancy and labor. Plasma AEA levels fell from the first to the second and third trimesters with no change between the second and third trimesters. The levels

rose at term before the onset of clinically apparent labor and rose further during labor to represent a 6-fold increase from third-trimester levels.

The studies of Maccarrone *et al.* (33) demonstrated that, in IVF pregnancies, low plasma AEA levels were associated with pregnancies that progressed beyond the first trimester of pregnancy, indicating that a successfully developing conceptus suppresses systemic levels of plasma AEA. That this suppression may be required for normal development of the fetus and pregnancy maintenance is supported by the association of high AEA levels with miscarriages (34). Endocannabinoids are also known to have adverse effects on successful pregnancy in mice (29, 37–39). High levels have been demonstrated to block the development of two-cell mouse embryos at the blastocyst stage and at blastocyst hatching (37), a mechanism that may be associated with pregnancy failure or growth restriction of early onset. The requirement for an environment with low levels of AEA is supported by the effects of exogenous cannabinoids, which act upon the endocannabinoid receptors, and which are associated with miscarriages, fetal growth restriction, and preterm labor (22–26).

Surprisingly, in our study the levels detected in the first trimester, which were similar to those reported in successful IVF pregnancies (33), were similar to those in the luteal phase of the menstrual cycle. This implies that the low levels of AEA proposed to be required to support early pregnancy are already established in the luteal phase, enabling successful implantation, and that successful pregnancy represents a successful maintenance of these suppressed levels. The low levels in postmenopausal women and the high levels in the follicular phase suggest that steroid hormones primarily regulate AEA levels, with estradiol increasing the levels and progesterone suppressing them. The effect of progesterone could result from regulation of the degradation of peripheral AEA by peripheral blood mononuclear cells given that the levels of FAAH, the principal enzyme involved in AEA degradation, in these cells are regulated by progesterone (40, 41). The induction of high AEA levels by estradiol could be mediated by its effect upon endothelial cells given that it has been reported that estradiol increases the release of AEA from these cells into the circulation (42).

However, these considerations do not account for the dramatic increase in the levels of AEA at term before the onset of clinically apparent labor and subsequently during labor when there are no changes in systemic steroid hormone levels. It is unlikely that the increase in AEA concentrations is due to pain because there was no correlation between AEA concentrations and the use of epidural analgesia during labor. Unless AEA production is uniquely stimulated by labor, the previous considerations of steroid hormone control of its production and degradation could be explicable if, as has been proposed for the uterus (43), a “functional progesterone withdrawal” occurs in peripheral blood mononuclear cells such that the induction of FAAH by progesterone fails, allowing AEA levels to rise. Although this seems a logical explanation, there are potentially many (yet unidentified) factors that may be involved in this process.

These observations beg the question as to the possible function of AEA in parturition and labor. Labor is associated

with an increased local production of prostaglandins (44) that act on all reproductive tissues and, given that AEA acts as a reservoir for arachidonic acid, the rise in AEA levels could be to provide a large reservoir of the precursor for prostaglandin production. Whether equivalent rises in AEA occur during preterm labor awaits further study. The cannabinoid receptors, CB1 and CB2, are present in the uterus (31), and *in vitro* stimulation of these receptors results in relaxation of myometrial strips (31), which appears paradoxical given the rise in AEA associated with labor in this study. However, it is possible that if the cannabinoid receptors are differentially expressed within the uterus, then AEA action could be associated with relaxation of myometrial cells within the lower uterine segment during labor. This interpretation may be supported by our observation that AEA levels rose at term before the onset of clinically apparent uterine contractions and labor, a period when development of the lower uterine segment and cervical dilation occur.

In conclusion, these observations underline the potential role of endocannabinoids in human pregnancy maintenance and labor. However, little is known about the mechanisms by which these cannabinoids affect these processes, and it is only through an understanding of these mechanisms that the importance of dysregulation of production and metabolism regulation of AEA can be understood. Until this is completely understood, we recommend that the current exploration of the use of exocannabinoids for pain relief in labor be delayed. Previous observations that the highest level of AEA ever measured in any tissue in the mouse was found in the uterus and its local effect on the contracting human pregnant myometrial strips (31) suggest a uterine origin, although there could potentially be other sources.

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