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Progesterone Up-Regulates Anandamide Hydrolase in Human Lymphocytes: Role of Cytokines and Implications for Fertility

Mauro Maccarrone,† Herbert Valensise,‡ Monica Bari,* Natalia Lazzarin,‡ Carlo Romanini,‡ and Alessandro Finazzi-Agrò*‡

Physiological concentrations of progesterone stimulate the activity of the endocannabinoid-degrading enzyme anandamide hydrolase (fatty acid amide hydrolase, FAAH) in human lymphocytes. At the same concentrations, the membrane-impermeant conjugate of progesterone with BSA was ineffective, suggesting that binding to an intracellular receptor was needed for progesterone activity. Stimulation of FAAH occurred through up-regulation of gene expression at transcriptional and translational level, and was partly mediated by the Th2 cytokines. In fact, lymphocyte treatment with IL-4 or with IL-10 had a stimulating effect on FAAH, whereas the Th1 cytokines IL-12 and IFN-γ reduced the activity and the protein expression of FAAH. Human chorionic gonadotropin or cortisol had no effect on FAAH activity. At variance with FAAH, the lymphocyte anandamide transporter and cannabinoid receptors were not affected by treatment with progesterone or cytokines. Good FAAH substrates such as anandamide and 2-arachidonoylglycerol inhibited the release of leukemia-inhibitory factor from human lymphocytes, but N-palmitoylethanolamine, a poor substrate, did not. A clinical study performed on 100 healthy women showed that a low FAAH activity in lymphocytes correlates with spontaneous abortion, whereas anandamide transporter and cannabinoid receptors in these cells remain unchanged. These results add the endocannabinoids to the hormone-cytokine array involved in the control of human pregnancy. The Journal of Immunology, 2001, 166: 7183–7189.

Endocannabinoids form an emerging class of lipid mediators, which includes amides and esters of long chain polyunsaturated fatty acids, found in several human tissues (1, 2). Anandamide (N-arachidonylethanolamine, AEA)3 and 2-arachidonoylglycerol (2-AG) are the main endocannabinoids described to date (2, 3). They bind to both brain (CB1) and peripheral (CB2) cannabinoid receptors, thus mimicking some of the psychotropic and analgesic effects of Δ9-tetrahydrocannabinol, the psychoactive principle of hashish and marijuana (1). AEA and 2-AG have cardiovascular activity, by inducing vasorelaxation (4, 5), and act as immune modulators (6, 7), much alike the exogenous cannabinoids (8, 9). Also, N-palmitoylethanolamine (PEA) is a bio logically active endocannabinoid, reported to have antiinflammatory activity (10). However, its ability to bind to CB receptors is still controversial (2). Recently, we reported on the association between decreased concentrations of the AEA-degrading enzyme, fatty acid amide hydrolase (FAAH), in peripheral lymphocytes and early pregnancy failure in humans (11). This finding seemed of interest, because the cellular and molecular mechanisms of spontaneous abortion remain largely unknown, yet about one-half of all conceptions are lost before the expected menses (12).

Progesterone (P), a hormone essential for the maintenance of pregnancy, is also known to modulate immune function (13) and to elicit an immunological response critical for normal gestation (14). Indeed, P has been shown to favor the development of human T lymphocytes producing Th2 cytokines (ILs 4 and 10), which inhibit Th1-type cytokines (IL-12 and IFN-γ), thus allowing the survival of fetal allograft and therefore a successful pregnancy (15, 16). More recently, the P-induced Th2 bias has been found to stimulate the release of LIF from T lymphocytes, mediated by IL-4 (17). Clinical data showing that women with unexplained recurrent abortions have a reduced LIF production suggest that the latter is indeed critical for implantation and maintenance of fetus in humans (17–19). In this study, we sought to investigate whether P stimulates FAAH activity and expression in human lymphocytes. This would provide a biochemical ground to our previous observation that low FAAH activity correlates with pregnancy loss in humans (11). Since AEA and related compounds bind to CB receptors, and their degradation by intracellular FAAH requires cellular uptake through a specific transporter (20, 21), the effect of P on CB receptors and FAAH transporter in lymphocytes was also investigated. Moreover, we studied the role of Th1- and Th2-type cytokines in the regulation of FAAH, to ascertain a possible correlation among the effects of P. We also investigated the effect of human chorionic gonadotropin (hCG) on FAAH activity, because of its role in pregnancy (22), and its use as a marker to monitor human gestation (23). Finally, we investigated whether AEA and its congeners might reduce LIF release from lymphocytes.

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Abbreviations used in this paper: AEA, anandamide (N-arachidonylethanolamine); AAOCEF, arachidonoylthreomethyl ketone; 2-AG, 2-arachidonoylglycerol; AM404, N-(4-hydroxyphenoxy)arachidonylamide; CAPS, capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide); CB1/2, type 1/2 cannabinoid; CP55.940, 5-(1′,3′-dimethylheptyl)-2-(1′RS,2′RS)-3-hydroxypropyl)cyclohexylidenepheno; FAAH, fatty acid amide hydrolase; GAR-AP, goat anti-rabbit Abs conjugated with alkaline phosphatase; hCG, human chorionic gonadotropin; IGF-IR, insulin-like growth factor 1 receptor; P, progesterone; P-BSA, P 3-(O-carboxyethyl)oxime/BSA conjugate; PEA, N-palmitoylethanolamine; RU486, mifepristone; rIL, soluble ILs; SR141716, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide; SR144528, N-[1(5)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide.
Materials and Methods

Reagents

Chemicals were of the purest analytical grade. AEA, P, 3-[(O-carboxy-ethyl)oxime]-BSA conjugate (P-BSA), mifepristone (RU486), hCG, hCG (17-hydroxy corticotropin), and human rIL-2 were purchased from Sigma (St. Louis, MO). N-(4-hydroxyphenyl)arachidonoylamine (AM404), arachidonyl-triolethanol amine containing ketone (AAOCOCF₃), and 2-arachidonylglycerol (2-AG) were from Research Biochemicals International (Natick, MA). Human hIL-4, hIL-10, hIL-12, human rIFN-γ, PMA, tetanus toxoid (Cholstridiun tetani), and capsaicin (N-(1)/(2)-(4-chlorophenyl)ethyl)-1,3,4,5-tetrahydro-2H-2-benzazepine-2-carbothioamide, CAPS) were from Calbiochem (La Jolla, CA). N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (SR144528) and N-(1/(5))endo-1,3,3-trimethylbicyclo[2.2.1] heptan-2-yl)-1-(4-(chloro-3-methylpheny1)1-(4-(4-methylbenzyl) pyrazole-3-carboxylic acid (SRI14176) and N-[(1)-endo,1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-(chloro-3-methylpheny1)-1-(4-(4-methylbenzyl)pyrazole-3-carboxylate (SRI145428) was a kind gift of Sanofi Recherche (Monpellier, France). PE was synthesized and characterized (purity > 96%) by gas-liquid chromatography, as reported (24). [3H]ABA (221 Ci/mmol) and [3H]CP55,940 (5.1-11-(dimethylethyl)-2-[18S,5S-hydroxy-2R-(3-hydroxypro pyl)cycholethyl]phenol, 126 Ci/mmol) were from NEN DuPont de Nemours (Cologne, Germany). 2-[(3H]JAG was synthesized from 1,3-dihexanoyl-2-propanol and [3H]arachidonic acid (200 Ci/mmol, ARC, St. Louis, MO), as reported (25). Anti-FAAH polyclonal Abs were elicited in rabbits against the peptide, with a FAAB sequence GYVEYTDNYTMAPSPAMR (26) conjugated to OVA (46.0 kDa). For immunochemical analysis, gels were electroblotted with anti-FAAH polyclonal Abs (1/200), using GAR-AP, diluted 1/2000, as second Ab (24). Densitometric analysis of filters was performed by means of a Floor-S Multimmer equipped with a Quantity One software (Bio-Rad). In some experiments, the RT-PCR products were excised from the gel and counted in a LKB1214 Rackbeta scintillation counter (Amer sham Pharmacu Biotech). Products were validated by size determination and sequencing. Linear amplification sequencing was performed by using Cyclist DNA Sequencing Kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. RT-PCR products for sequencing were prepared without the [α-32P]dCTP and sequenced with the same primers used for amplification, after labeling them with [α-32P]dATP (300 Ci/mmol; Amersham Pharmacu Biotech).

Materials and Methods

Isolation and treatment of lymphocytes

Blood samples (20 ml/donor) were drawn from the antecubital vein of healthy nonpregnant women (age range 28–35 years), who gave informed consent to the study, and were collected into heparinized sterile tubes. All subjects had regular cycles, and sampling was performed between the seventh and the tenth day from the last menstrual period. Peripheral lymphocytes (3 × 10⁸) were generated from human lymphocytes, as described (15). Peripheral lymphocytes (10⁶ cells/ml, in ventilated 25-ml flasks. Incubation of lymphocytes (3 × 10⁸ cells/ml) with [3H]CP55,940 (400 mCi/mmol) was then added to the indicated concentration, and for the indicated periods of time. Controls were incubated for 1 h; then heat-inactivated FBS (Life Technologies) was added at a final concentration of 10% (24). Cell viability after each treatment was tested by trypan blue dye exclusion, and was found to be higher than 90% in all cases.

AEA hydroxylase activity and expression

FAAH (EC 3.5.1.4) activity was assayed at pH 9 with 10 μM [3H]AEA as substrate, by the reversed-phase HPLC method previously described (24). In a preliminary series of experiments, T cells were isolated from the other PBMCs by means of the Dynal CD2 CELLection kit (Dynal, Olso, Norway), according to the manufacturer’s instructions. It was found that FAAH activity in lymphocytes accounts for 85% of the activity measured in the lymphocyte medium. This activity was measured in the RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 25 mM HEPES, 2.5 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (24), at a density of 1.5 × 10⁶ cells/ml, in ventilated 25-ml flasks. Incubation of lymphocytes with P, alone or in the presence of different compounds, with cytochexin, with hCG, with hCG, or with anti-cannabinoid antibodies, was performed at 37°C in humidified 5% CO₂ atmosphere, at the indicated concentrations and for the indicated periods of time. Controls were incubated with vehicles alone. In all cases, cells were treated in serum-free medium for 1 h; then heat-inactivated FBS (Life Technologies) was added at a final concentration of 10%. Cell viability after each treatment was tested by trypan blue dye exclusion, and was found to be higher than 90% in all cases.

Analysis of AEA uptake and cannabinoid receptors

The uptake of [3H]AEA by intact lymphocytes (2 × 10⁶/test) was studied essentially as described (21). To discriminate non-carrier-mediated from carrier-mediated transport, through cell membranes, the density separation medium Lymphoprep (Nycomed Pharma, Oslo, Norway), as reported (24). Purified lymphocytes were resuspended in RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 25 mM HEPES, 2.5 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (24), at a density of 1.5 × 10⁶ cells/ml, in ventilated 25-ml flasks. Incubation of lymphocytes with P, alone or in the presence of different compounds, with cytochexin, with hCG, with hCG, or with anti-cannabinoid antibodies, was performed at 37°C in humidified 5% CO₂ atmosphere, at the indicated concentrations and for the indicated periods of time. Controls were incubated with vehicles alone. In all cases, cells were treated in serum-free medium for 1 h; then heat-inactivated FBS (Life Technologies) was added at a final concentration of 10% (24). Cell viability after each treatment was tested by trypan blue dye exclusion, and was found to be higher than 90% in all cases.

Release of LIF

To induce LIF production, short-term tetanus toxoid-specific T cell cultures were generated from human lymphocytes, as described (15). Peripheral lymphocytes (3 × 10⁶/test) were stimulated for 5 days with tetanus toxoid (0.2 μg/ml), in the absence or presence of AEA, 2-AG, PEA, SR141716, SR144528, CAPS, AM404, AAOCOCF₃, or different combinations of them, at the indicated concentrations. Human IL-2 (20 U/ml) was then added to all cultures, which were continued for additional 9 days. On day 14, T cell blasts were stimulated with PMA (20 ng/ml) plus anti-CD3 mAbs (100 ng/ml), and 36 h later culture supernatants were collected for LIF assay (17). LIF was quantitated by the QuantiKine human LIF immunoassay, according to the manufacture’s instructions. OD₄₅₀ nm values of unknown samples were converted into LIF concentrations by calibration curves with human LIF (linearity range 10–1000 pg/ml).

Clinical study

A cohort of 100 white Italian women (age range 28–35 years) was enrolled after obtaining informed consent, and was selected from those who applied for antenatal booking at the outpatient clinic of San Giovanni Calibita Hospital. Women had normal singleton pregnancies, and embryos with crown-rump lengths of > 10 mm and a heart rate of > 110 beats/min. We included women enrolled from enrollment patients with chronic diseases (diabetes, hyper tension, lupus erythematosus systemicus), those with a known history of recurrent spontaneous abortions, those with uterine anomalies, those under chronic ongoing treatments (corticosteroids, low dose aspirin), and those...
who smoked >20 cigarettes per day. We also excluded women with any sign of vaginal bleeding, abdominal pain, or any other abnormal sign. Women had repeated ultrasonographs and a single blood test at gestational ages between 7 and 8 wk, and were all observed until 13 wk of gestation. Pregnant women were then examined at 22 and 32 wk of gestation. Gestational age was assessed on the basis of the date of the last normal menstrual period, confirmed through the first crown-rump length measurement. Spontaneous (missed) abortion was defined as the absence of cardiac activity in the embryo, assessed by ultrasonography. All the ultrason examinations were performed using a 6.5-MHz transvaginal probe and a 3.5-MHz transabdominal probe (AU4 IDEA Esaote, Ansaldo, Milan, Italy). Lymphocyte isolation and assays of FAAH activity, of AEA uptake, and of CP55,940 binding were performed as described above for the in vitro studies. There were no differences in phenotype among lymphocyte preparations used in this study, especially between normal and aborted pregnancies.

Statistical analysis
Data reported in this work are the mean (±SD) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney test, elaborating experimental data by means of the InStat program (GraphPad, San Diego, CA).

Results

$P$ stimulates FAAH activity and expression in human lymphocytes

In vitro treatment of human lymphocytes with $P$ enhanced their FAAH activity in a dose-dependent manner, while $P$ conjugated with BSA was ineffective in the same concentration range (Fig. 1A). FAAH activation reached statistical significance ($p < 0.05$) at 250 nM $P$ and a maximum at 1 μM. Therefore, the last concentration was chosen to further investigate the effect of $P$ on FAAH. Time-course experiments showed that $P$-induced activation of FAAH was significant ($p < 0.05$) 12 h after lymphocyte treatment and reached a maximum at 24 h (Fig. 1B). Western blot analysis of lymphocyte extracts showed that specific anti-FAAH Abs recognized a single immunoreactive band of the molecular size expected for FAAH, the intensity of which increased dose dependently in P-treated cells (Fig. 2A). Densitometric analysis of the filter shown in Fig. 2A (representative of triplicate experiments) indicated that FAAH protein increased to 130, 160, or 220% of the control (100% = 10,000 ± 1,000 U/mm²), in cells treated for 24 h with 250, 750, or 1,000 nM $P$, respectively. The same anti-FAAH Abs were used to quantify FAAH content by ELISA, showing that $P$ increased time dependently FAAH protein in human lymphocytes in parallel to the increase of enzymic activity (Fig. 1B). Kinetic analysis showed that FAAH activity toward AEA had apparent maximum velocity ($V_{max}$) values of 190 ± 20 and 380 ± 35 pmol/min/mg protein, in homogenates of lymphocytes treated for 24 h with either vehicle or 1 μM $P$, whereas the apparent Michaelis-Menten constant ($K_m$) was unchanged (8 ± 1 μM in both cases). Moreover, FAAH in untreated lymphocytes was also able to hydrolyze 2-AG with apparent $K_m = 7 ± 1 μM$ and $V_{max} = 230 ± 25$ pmol/min/mg protein, whereas PEA was hydrolyzed with apparent $K_m = 15 ± 2 μM$ and $V_{max} = 50 ± 5$ pmol/min/mg protein. RT-PCR amplification of cDNA of human lymphocytes showed a single band of the expected molecular size for FAAH gene, which increased dose dependently in P-treated cells (Fig. 2B). Densitometric analysis of the autoradiographic film shown in Fig. 2B (representative of triplicate experiments) indicated that FAAH mRNA increased to 120, 155, or 190% of the control (100% = 2,700 ± 300 U/mm²), in cells treated for 24 h with 250, 750, or 1,000 nM $P$, respectively. Under the same experimental conditions, the expression of the 18S rRNA gene was unaffected (Fig. 2B). Liquid scintillation counting of RT-PCR products showed that $P$ increased...
time dependently FAAH mRNA in human lymphocytes, in a way parallel to that of enzymic activity and protein content (Fig. 1B). *Th2 cytokines up-regulate and Th1-type cytokines down-regulate FAAH*

The mechanism of FAAH up-regulation by P was investigated by using different substances. In pilot experiments, various doses of each compound were used, and the results obtained with the most effective concentrations are shown in this study. Treatment of human lymphocytes (3 × 10⁶/test) with Th2-type cytokines IL-4 (5 ng/ml) or IL-10 (1 ng/ml) for 24 h increased FAAH activity up to 230% or 180% of the control, respectively, whereas treatment with Th1-type cytokines IL-12 (5 ng/ml) or IFN-γ (0.2 ng/ml) reduced FAAH activity to approximately 50% of the control (Fig. 3A).

**FIGURE 3.** Effect of cytokines on FAAH activity and content. A. Effect of Th2-type (IL-4, 5 ng/ml, and IL-10, 1 ng/ml) or Th1-type (IL-12, 5 ng/ml, and IFN-γ, 0.2 ng/ml) cytokines on FAAH activity (□) and protein content (□) in human lymphocytes, after 24 h of incubation (100% = 140 ± 15 pmol/min/mg protein for the activity, or 0.220 ± 0.025 OD units at 405 nm for the protein content). B. Effect of P (1 μM = 0.3 μg/ml), alone or in the presence of sIL-4R (0.25 μg/ml), Abs against IL-4R (0.25 μg/ml), “mock” Abs against IGF-IR (0.25 μg/ml), or RU486 (14 μM = 6 μg/ml), on FAAH activity (□) and protein content (□), after 24 h of incubation (100% as in A). In both panels, bars represent SD values. *p < 0.01 vs control. In B, **p < 0.05 vs control; @, p > 0.05 vs control; #, p < 0.05 vs P-treated lymphocytes; ***p > 0.05 vs P-treated lymphocytes; §, p < 0.01 vs P-treated lymphocytes.

FAAH expression at the protein level, measured by ELISA, paralleled the enzyme activity (Fig. 3A).

The possible link between P and Th2-type cytokines in enhancing FAAH activity and expression was investigated by co-incubating human lymphocytes (3 × 10⁶/test) for 24 h with 1 μM P (0.3 μg/ml), alone or with sIL-4R (0.25 μg/ml), or with anti-IL-4R Abs (0.25 μg/ml), or with anti-IGF-IR (mock) Abs (0.25 μg/ml), as reported (17). Both sIL-4R and anti-IL-4R Abs, but not mock Abs, significantly (p < 0.05) reduced FAAH activation by P, although it remained significantly (p < 0.05) higher than FAAH activity and expression in the untreated controls (Fig. 3B). Finally, RU486, a synthetic antiprogestin compound (30), fully reverted the effect of 1 μM P on FAAH activity and expression (Fig. 3B), when used at a concentration of 14 μM (=6 μg/ml) (Fig. 3B). Instead, hCG did not significantly (p > 0.05) affect FAAH activity in human lymphocytes, at concentrations up to 400 IU/ml; neither did 2 μM cortisol.

**P and cytokines do not affect AEA uptake and cannabinoid receptor binding**

Intact lymphocytes were able to accumulate [³H]AEA in a temperature (Q₁₀ = 1.6)-, time (t₁/₂ = 5 min)-, and concentration-dependent manner (data not shown), according to a saturable process with apparent Kₘ = 130 ± 15 nM and Vₘₐₓ = 75 ± 8 pmol/min/mg protein. The uptake of 200 nM [³H]AEA was almost completely inhibited by 10 μM AM404 (Table I), a specific AEA transport inhibitor (31). However, treatment of lymphocytes (3 × 10⁶/test) for 24 h with P, IL-4, IL-10, IL-12, IFN-γ, hCG, or cortisol did not quite affect [³H]AEA uptake (Table I).

Human lymphocytes were able to bind [³H]CP55,940, a synthetic cannabinoid that binds with high affinity both CB1 and CB2 receptors (32). The selective CB1R antagonist SR141716, but not the selective CB2R antagonist SR144528 (32), both used at 0.1 μM, displaced [³H]CP55,940, suggesting that only CB1R was exacerbed on the lymphocyte surface (Table II). Treatment of lymphocytes (20 × 10⁶/test) for 24 h with P, IL-4, IL-10, IL-12, IFN-γ, hCG, or cortisol did not affect [³H]CP55,940 binding (Table II).

**Endocannabinoids inhibit the release of LIF in a CB1R-dependent manner**

Treatment of human lymphocytes (3 × 10⁶/test) with 0.5 μM AEA reduced the production of LIF to 33% of the untreated control (Table III). This effect of AEA was counteracted by 0.1 μM SR141716, but not by 0.1 μM SR144528 or by 1 μM CAPS, a selective antagonist of vanilloid receptors (33), suggesting that it

### Table I. Uptake of AEA by human lymphocytes treated with hormones or cytokines

<table>
<thead>
<tr>
<th>Compound Added to Lymphocytes</th>
<th>[³H]AEA Uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>AM404 (10 μM)</td>
<td>20 ± 2*</td>
</tr>
<tr>
<td>Progesterone (1 μM)</td>
<td>105 ± 10**</td>
</tr>
<tr>
<td>IL-4 (5 ng/ml)</td>
<td>95 ± 10**</td>
</tr>
<tr>
<td>IL-10 (1 ng/ml)</td>
<td>90 ± 10**</td>
</tr>
<tr>
<td>IL-12 (5 ng/ml)</td>
<td>95 ± 10**</td>
</tr>
<tr>
<td>IFN-γ (0.2 mg/ml)</td>
<td>100 ± 10**</td>
</tr>
<tr>
<td>hCG (400 IU/ml)</td>
<td>100 ± 10**</td>
</tr>
<tr>
<td>Cortisol (2 μM)</td>
<td>100 ± 10**</td>
</tr>
</tbody>
</table>

*The ability of human lymphocytes to take up [³H]AEA was determined 24 h after treatment with each compound and was expressed as percentage of the untreated control (100% = 50 ± 5 pmol/min/mg protein). *p < 0.01 vs the untreated control; **p > 0.05 vs the untreated control.
was mediated by CB1 receptors only (Table III). On the other hand, coincubation of lymphocytes with 0.5 mM AEA and 10 mM AM404 or 10 mM AACOCF3, a FAAH inhibitor (34), further reduced the LIF release to 17% of the untreated controls (Table III).

2-AG also reduced LIF production by lymphocytes to 42% of the untreated control, whereas twice concentrated PEA was ineffective (Table III).

FAAH activity, AEA uptake, and CBR binding in patients

In a clinical study, we measured FAAH activity, [3H]AEA uptake, and [3H]CP55.940 binding in lymphocytes isolated from 100 healthy women at 7–8 wk of gestation. This is the earliest time point in gestation in which the difference between FAAH content in women who miscarried and those who did not was found to be significant (11). Ultrasonography showed that approximately 10 days after the blood tests, 15 subjects miscarried and 85 did not. The a posteriori association between the gestation outcome and FAAH activity, AEA uptake, or CP55.940 binding showed that FAAH activity (Fig. 4A) was lower in all the 15 women who miscarried than in the 85 who did not (48 ± 6 vs 133 ± 8 pmol/min/mg protein; p, 0.0001), whereas AEA uptake (49 ± 4 vs 50 ± 4 pmol/min/mg protein; p > 0.05) and CP55.940 binding (20,400 ± 1,795 vs 20,380 ± 1,930 cpm/mg protein; p > 0.05) were similar in both groups (Fig. 4, B and C).

Discussion

It is well known that a rise in P is linked to a normal evolution of pregnancy. In this study, we show that P stimulates FAAH activity in human lymphocytes, at the concentrations found in serum during pregnancy (from 0.02 to 0.30 μg/ml) (see Ref. 15 and refer-

Table II.  **Binding of CP55.940 by human lymphocytes treated with hormones or cytokines**

<table>
<thead>
<tr>
<th>Compound Added to Lymphocytes</th>
<th>[3H]CP55.940 Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>SR141716 (0.1 μM)</td>
<td>20 ± 2*</td>
</tr>
<tr>
<td>SR144528 (0.1 μM)</td>
<td>90 ± 10**</td>
</tr>
<tr>
<td>Progesterone (1 μM)</td>
<td>105 ± 10**</td>
</tr>
<tr>
<td>IL-4 (5 ng/ml)</td>
<td>95 ± 10**</td>
</tr>
<tr>
<td>IL-10 (1 ng/ml)</td>
<td>90 ± 10**</td>
</tr>
<tr>
<td>IL-12 (5 ng/ml)</td>
<td>95 ± 10**</td>
</tr>
<tr>
<td>IFN-γ (0.2 ng/ml)</td>
<td>100 ± 10**</td>
</tr>
<tr>
<td>hCG (400 IU/ml)</td>
<td>100 ± 10**</td>
</tr>
<tr>
<td>Cortisol (2 μM)</td>
<td>100 ± 10**</td>
</tr>
</tbody>
</table>

* The ability of human lymphocytes to bind [3H]CP55.940 was determined 24 h after treatment with each compound and was expressed as percentage of the untreated control (100% = 20,000 ± 2,000 cpm/mg protein). *, p < 0.01 vs the untreated control; **, p > 0.05 vs the untreated control.

Table III. **Effect of endocannabinoids on LIF release by human lymphocytes**

<table>
<thead>
<tr>
<th>Compound Added to Lymphocytes</th>
<th>LIF Release (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>120 ± 24 (100%)</td>
</tr>
<tr>
<td>AEA (0.5 μM)</td>
<td>40 ± 8 (33%)*</td>
</tr>
<tr>
<td>AEA (0.5 μM) + SR141716 (0.1 μM)</td>
<td>110 ± 22 (92%)*$</td>
</tr>
<tr>
<td>AEA (0.5 μM) + SR144528 (0.1 μM)</td>
<td>44 ± 8 (37%)*#</td>
</tr>
<tr>
<td>AEA (0.5 μM) + CAPS (1 μM)</td>
<td>42 ± 8 (35%)*</td>
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<tr>
<td>AEA (0.5 μM) + AM404 (10 μM)</td>
<td>20 ± 4 (17%)*</td>
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<tr>
<td>AEA (0.5 μM) + AACOCF3 (10 μM)</td>
<td>20 ± 4 (17%)*</td>
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<tr>
<td>2-AG (0.5 μM)</td>
<td>50 ± 10 (42%)*</td>
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<tr>
<td>PEA (1 μM)</td>
<td>110 ± 22 (92%)*$</td>
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</table>

*, p < 0.01 vs the untreated controls; **, p > 0.05 vs the untreated controls; #, p > 0.05 vs AEA-treated cells; $, p < 0.01 vs AEA-treated cells.
gene. This effect can be either direct, i.e., through binding of the P/PR complex to the FAAH gene promoter, or indirect, i.e., through P/PR-dependent production of a transcription factor that binds to the promoter. In this context, it seems noteworthy that a specific steroid/steroid receptor motif has been recently described in the promoter region of FAAH gene (36), which may suggest a direct effect of the P/PR complexes on FAAH transcription. On the other hand, the effect of RU486 (Fig. 3B), a PR antagonist that stabilizes PR in a form unable to bind DNA (30), is compatible with both hypotheses. Remarkably, RU486 was effective at a concentration known to modulate immunoendocrine interactions in early pregnancy of humans (37). At any rate, the up-regulation of FAAH expression by P is a major finding of this investigation, associated with higher FAAH activity.

During pregnancy, P stimulates T lymphocytes to produce Th2-type cytokines (IL-4 and IL-10), but lowers the production of Th1-type cytokines (IL-12 and IFN-γ) (13, 15, 16). In this study, we show that IL-4 and IL-10 stimulated the activity and protein expression of lymphocyte FAAH, which were instead inhibited by IL-12 and IFN-γ (Fig. 3A). Moreover, IL-4, which is known to mediate the favorable effects of P on pregnancy (14–17), also mediated the effect of P on FAAH. In fact, the FAAH up-regulation was significantly lower when IL-4 was sequestered by sIL-4R or by anti-IL-4R Abs (Fig. 3B). However, FAAH activity and protein content remained significantly higher in lymphocytes treated with P in the presence of sIL-4R or anti-IL-4R than in the untreated controls, suggesting that IL-4 enhanced, but was only in part responsible for the effects of P. Physiological concentrations of hCG were ineffective on FAAH activity, which is in keeping with the observation that the levels of hCG do not always correlate with the outcome of pregnancy (22, 23). Consistently, the same concentrations of hCG used in this study failed to modulate Th1/Th2-type cytokine production by human lymphocytes (15). Also the glucocorticoid cortisol failed to induce FAAH, ruling out that P might act through glucocorticoid receptors (38).

Human lymphocytes have a specific AEA transporter, and its affinity for AEA is very close to that of human platelets (39), and identical to that of human lymphoma (24) and endothelial cells (21). Therefore, it can be proposed that the same carrier is present on the surface of these different human blood cells. Interestingly, P, IL-4, IL-10, IL-12, IFN-γ, hCG, or cortisol under the same conditions tested for FAAH activity did not affect AEA transport (Table I). This finding suggests that the transport was not a “checkpoint” for AEA degradation during pregnancy. This concept is in keeping with the notion that AEA is taken up through a facilitated diffusion mechanism (20, 21, 24, 27) that is driven by the AEA gradient between the outer and inner side of the plasma membrane, maintained also by FAAH activity. Lymphocytes express CB1 receptors, which, however, are not involved in the up-regulation of FAAH by P (Table II). Indeed, P, IL-4, IL-10, IL-12, IFN-γ, hCG, or cortisol were ineffective on CB receptors of lymphocytes, supporting the concept that degradation by FAAH is the only critical event in controlling the endocannabinoid level during gestation. The clinical study, showing that FAAH activity was defective in lymphocytes of women who miscarried compared with those who did not (Fig. 4A), whereas AEA transporter and CB receptors were the same in both groups (Fig. 4, B and C), is a further evidence for the critical role of this enzyme. Moreover, data reported in Fig. 4A extend to enzyme activity our recent observation on FAAH protein content in women who miscarried compared with those who did not (11).

High FAAH activity should lower the level of its substrates. Since it has been shown that peripheral lymphocytes play a critical role in human pregnancy by producing LIF (17, 18), we tested whether the endocannabinoids would affect LIF release from peripheral T cells. The results, summarized in Table III, show that indeed AEA reduced LIF release from lymphocytes in a CB1 receptor-mediated manner, and that increasing the extracellular concentration of AEA by blocking its uptake or hydrolysis enhanced this effect. In this context, it should be recalled that AACOCF3 is a powerful inhibitor of FAAH, which can also inhibit phospholipase A2 (34), leaving open the possibility that this latter enzyme might be involved in the effect of AEA on LIF release. However, the identity between the effect of AACOCF3 and that of AM404, a specific inhibitor of the FAAH transporter (31), suggests that blockade of AEA degradation was the critical step, making it unlikely that other unrelated pathways might be involved. Moreover, the lack of effect of CAPS rules out that AEA might reduce LIF release acting through vanilloid receptors (32). Also, 2-AG inhibited LIF production, while PEA did not (Table III). 2-AG, like AEA, binds to CB1 receptors (2, 3) and can be hydrolyzed by lymphocyte FAAH as much as AEA (11). On the other hand, PEA was hydrolyzed by lymphocyte FAAH 10-fold less efficiently than AEA, and its ability to bind to CB receptors and to have anti-inflammatory actions in humans is still controversial (2, 28). Altogether, these data suggest that a low FAAH activity, and hence higher AEA and 2-AG levels, can lead to spontaneous abortion by reducing LIF production. This unprecedented effect of AEA is consistent with its adverse effects on embryo implantation and development in mouse (40–44). Moreover, keeping in mind the role of LIF in regulating growth and differentiation of neurons and endothelial cells (19), a wider implication of the present findings can be anticipated. The interplay among P, cytokines, FAAH, endocannabinoids, and LIF is depicted in Fig. 5. It is shown that P, by interacting with its receptor, increases the synthesis of FAAH, which in turn reduces the extracellular concentration of AEA by driving its import through the transporter. In this way, the effect of AEA on LIF release by binding to type 1 cannabinoid receptors is reduced. FAAH activation by P is further enhanced by IL-4. This cytokine can also directly activate FAAH, as does IL-10, whereas IL-12 or IFN-γ inhibit FAAH activity.
References
mediated by endothelial anandamide receptors. Hypertension 33:429.
cannabinoid anandamide potentiates interleukin-6 production by astrocytes in-
jected with Theiler’s murine encephalomyelitis virus by a receptor-mediated 
interleukin-2 by the putative endogenous cannabinoid 2-arachidonoylglycerol is 
mediated through down-regulation of the nuclear factor of activated T cells. Mol. 
cannabinol and cannabinol alter cytokine production by human immune cells. 
Immunopharmacology 40:179.
11. Maccarrone, M., H. Valensise, M. Bari, N. Lazzarin, C. Romanini, and A. Fi-
nazzi-Agro. 2000. Relationship between decreased anandamide hydrolysis concentra-
immunological pregnancy protective effect of progesterone is manifested via con-
cytokines and promotes both IL-4 production and membrane CD30 expression in 