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

J Immunol 2001; 166:7183-7189; doi: 10.4049/jimmunol.166.12.7183
http://www.jimmunol.org/content/166/12/7183

References
This article cites 43 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/166/12/7183.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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-Agro ✡,*

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 choric 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 amidases 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 psychotopic 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 biologically 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 (IL-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 AEA 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 choric 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.

*Department of Experimental Medicine and Biochemical Sciences and † Division of Obstetrics and Gynecology, University of Rome Tor Vergata, Rome, Italy; and ‡ Fatebenefratelli Association for Research, San Giovanni Calibita Hospital, Rome, Italy.

Received for publication November 27, 2000. Accepted for publication April 10, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was partly supported by Istituto Superiore di Sanità (III AIDS Program) and Ministero dell’Università e della Ricerca Scientifica e Tecnologica (PRIN Program), Rome, Italy.

2 Address correspondence and reprint requests to Dr. Alessandro Finazzi-Agro, Department of Experimental Medicine and Biochemical Sciences, University of Rome Tor Vergata, Via di Tor Vergata 135, I-00133 Rome, Italy. E-mail address: Finazzi@uniroma2.it

3 Abbreviations used in this paper: AEA, anandamide (N-arachidonylethanolamine); AACOCF₃, arachidonoyl trifluoroacetamide; 2-AG, 2-arachidonoylglycerol; AM404, N-[4-(hydroxyphenyl)arachidonoylamide]; CAPS, capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepin-2-carbothioamide); CB1/2, type 1/2 cannabinoid; CP55,940, 5-(1,1′-dimethylheptyl)-2-(1R,5R)-hydroyxycycloheptyliden; 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 I receptor; P, progesterone; P-BSA, P-[3-(O-carboxy methoxy)oxime]-BSA conjugate; PEA, N-palmitoylethanolamine; RU486, mifepristone; sIL, soluble IL; SR141716, N-(piperidin-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.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00

0222-1767/01/502.00
Materials and Methods

Reagents

Chemicals were of the purest analytical grade. AEA, P3-(O-carboxymethyl)oxime:BSA conjugate (P-BSA), mifepristone (RU486), hCG, HC(3-17-hydroxy-20-cortisone), and human rIL-2 were purchased from Sigma (St. Louis, MO), N-(4-hydroxyphenyl)arachidonoylamide (AM404), arachidonoylthromboxane methyl ketone (AACOCF3), and 2-arachidonoylglycerol (2-AG) were from Research Biochemicals International (Natick, MA). Human rIL-4, rIL-10, rIL-12, human rIFN-γ, PMA, tetanus toxoid (Choristidium tetani), and capsaicin (N-(1)-[2-(4-chlorophenyl)-ethyl]yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzenyl)pyrazole-3-carboxamide (SR144528) were a kind gift of Sanofi Recherche (Montpellier, France). PE was synthesized and characterized (purity >96% by gas-liquid chromatography), as reported (24). [3H]AEA (223 Ci/mmol) and [3H]CP55,940 (5.1-1.1 dimethylethyl)-2-[15-R,5-Hydroxy-2R-(3-hydroxypropyl)cyclohexyl]phenol, 126 Ci/mmol) were from NEN Du Pont de Nemours (Cologne, Germany). [3H]HAG was synthesized from l,3, dihexenyl-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 toxin, with the FAAH sequence VGYETDNTPMSAPM (26) conjugated to OVA, and were prepared by Primm (Milan, Italy). Anti-human CD3 mAbs were from Calbiochem. Soluble IL-4 (sIL-4), human rIL-1β, anti-human IL-4R (anti-IL-4R), anti-human insulin-like growth factor I receptor (anti-IGF-IR) mAbs, and Quantikine human LIF colorimetric sandwich ELISA were from R&D Systems (Minneapolis, MN). Goat anti-rabbit Abs conjugated with alkaline phosphatase (GAR-AP) were from Amersham Pharmacia Biotech (Little Chalfont, UK). RTP-PCR was performed using total RNA isolated from human lymphocytes (10×10^6 cells) by means of the Dynal CD2 CELLection kit (Dynal, Oslo, Norway), according to the manufacturer’s instructions. It was found that FAAH mRNA in a small number of lymphocytes accounted for up to 5% of the activity measured in the exponential phase of amplification of each gene. Briefly, 100 ng total RNA, for the amplification of FAAH, or 0.4 ng, for 18S rRNA, were reversibly transcribed and amplified in the same tube in a total reaction volume of 10 μl, in the presence of 3 μCi of [α-32P]dCTP (3000 Ci/mmol; Amer sham Pharmacia Biotech). The amplification parameters were as follows: 2 min at 95°C, 45 s at 95°C, 30 s at 55°C, and 30 s at 60°C. Linear amplification was observed after 20 cycles. The primers were as follows: (+), 5’-TGGAAAGTCTCGAACAGGGCCAG, and (−), 5’-TGTCATCA GACAGCCCTCAG, for FAAH; (+), 5’-AGTGTGCCGATTTA AAAAG, and (−), 5’-CCTCACGTTCCGAAAACAAA, for 18S rRNA. Five microliters of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography (24). The autoradiographic films were subjected to densitometric analysis, by means of a Floor-S Multimag equipped with a Quantity One software (Bio-Rad). In some experiments, the RTP-PCR products were excised from the gel and counted in a LKB1214 Rackbeta scintillation counter (Amer sham Pharmacia 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 (3000 Ci/mmol; Amer sham Pharmacia Biotech).

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 were purified by gradient centrifugation, using the density separation medium PyroPhase (20×10^6 cells/ml), which were then reacted with anti-FAAH polyclonal Abs (2 μg/ml), and were used in all experiments. For experiments conducted at 4°C, OD450 values could not be converted into FAAH concentrations, because the purified enzyme is not available to make calibration curves. However, the ELISA test was linear in the range 0–50 μg/well of cell homogenate, and its specificity for FAAH was confirmed by agar competition experiments (24). RT-PCR was performed using total RNA isolated from human lymphocytes (10×10^6 cells) by means of the simple nucleic acid preparation. Total RNA Isolation Kit (Invitrogen, Carlsbad, CA), as described (24). RT-PCR reactions were performed using the EZ 10th RNA PCR kit (Perkin-Elmer, Norwalk, CT), according to the manufacturer’s instructions. The reaction conditions were carefully examined to stop the reaction during the exponential phase of amplification of each gene. Briefly, 100 ng total RNA, for the amplification of FAAH, or 0.4 ng, for 18S rRNA, were reversibly transcribed and amplified in the same tube in a total reaction volume of 10 μl, in the presence of 3 μCi of [α-32P]dCTP (3000 Ci/mmol; Amer sham Pharmacia Biotech). The amplification parameters were as follows: 2 min at 95°C, 45 s at 95°C, 30 s at 55°C, and 30 s at 60°C. Linear amplification was observed after 20 cycles. The primers were as follows: (+), 5’-TGGAAAGTCTCGAACAGGGCCAG, and (−), 5’-TGTCATCA GACAGCCCTCAG, for FAAH; (+), 5’-AGTGTGCCGATTTA AAAAG, and (−), 5’-CCTCACGTTCCGAAAACAAA, for 18S rRNA. Five microliters of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography (24). The autoradiographic films were subjected to densitometric analysis, by means of a Floor-S Multimag equipped with a Quantity One software (Bio-Rad). In some experiments, the RTP-PCR products were excised from the gel and counted in a LKB1214 Rackbeta scintillation counter (Amer sham Pharmacia 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 (3000 Ci/mmol; Amer sham Pharmacia Biotech).

Analysis of AEA uptake and cannabinoid receptors

The uptake of [3H]AEA by intact lymphocytes (2×10^6/test) was analyzed essentially as described (21). To discriminate non-carrier-mediated from carrier-mediated transport through cell membranes, different concentrations of FAAH (50–1000 nM, as reported (28), were quickly frozen in liquid nitrogen, and stored at −80°C for no longer than 1 wk. Membrane fractions were used in rapid filtration assays with the synthetic cannabinoid [3H]CP55,940 at 400 PM, as described previously (28). Unspecific binding was determined in the presence of 10 μM AEA (29).

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 (3×10^6/test) were stimulated for 5 days with tetanus toxoid (0.2 μg/ml), in the absence or presence of AEA, 2-AG, PEA, SR144528, CAPS, AM404, AACOCF3, 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 manufacturer’s instructions. OD405 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 by crown-rump lengths of >10 mm and a heart rate of >110 beats/min. We enrolled 20% of patients with chronic diseases (diabetes, hypertension, 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% = 2700 ± 300 U/mm³), in cells treated for 24 h with 250, 750, or 1000 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 × 10^6/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).

**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 coincubating human lymphocytes (3 × 10^6/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-4 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 antiprogestinic 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 (Q_10 = 1.6), time (t_1/2 = 5 min), and concentration-dependent manner (data not shown), according to a saturable process with apparent K_m = 130 ± 15 nM and V_max = 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 × 10^6/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 expressed on the lymphocyte surface (Table II). Treatment of lymphocytes (20 × 10^6/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 × 10^6/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
was mediated by CB1 receptors only (Table III). On the other hand, coincubation of lymphocytes with 0.5 μM AEA and 10 μM AM404 or 10 μM 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 v 3 3 ± 6 pmol/min/mg protein; p, 0.0001), whereas AEA uptake (49 ± 6 v 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 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>
</tr>
<tr>
<td>AEA (0.5 μM) + AM404 (10 μM)</td>
<td>20 ± 4 (17%)*</td>
</tr>
<tr>
<td>AEA (0.5 μM) + AACOCF3 (10 μM)</td>
<td>20 ± 4 (17%)*</td>
</tr>
<tr>
<td>2-AG (0.5 μM)</td>
<td>50 ± 10 (42%)*</td>
</tr>
<tr>
<td>PEA (1 μM)</td>
<td>110 ± 22 (92%)*</td>
</tr>
</tbody>
</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.

FIGURE 4. Clinical study. FAAH activity (A), AEA uptake (B), and CP55.940 binding (C) in lymphocytes isolated from women with normal gestation (○) or spontaneous abortion (●). Biochemical data were associated a posteriori with the outcome of pregnancy.
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 immunoenocrine 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 AAOCFC2 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 AAOCFC2 and that of AM404, a specific inhibitor of the FAAH transporter (31), suggests that blockade of FAAH 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 FAAH 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 antiinflammatory actions in humans is still controversial (2, 28). Altogether, these data suggest that in 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 described 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.
Our results give a biochemical ground to our observation that low FAAH activity correlates with spontaneous abortion in humans (11). They represent the first evidence of a link between the hormone-cytokine network responsible for successful pregnancy and the peripheral endocannabinoid system, and suggest that FAAH, but not AEA transporter or CB receptors, might be critical for this link. These results might represent also a useful framework for the interpretation of a novel interaction between P and exogenous cannabinoids, recently shown to regulate female sexual receptivity (45).

References