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Progesterone Activates Fatty Acid Amide Hydrolase (FAAH) Promoter in Human T Lymphocytes through the Transcription Factor Ikaros

EVIDENCE FOR A SYNERGISTIC EFFECT OF LEPTIN*

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Physiological concentrations of progesterone stimulate the activity of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) in human T lymphocytes, up to a ~270% over the untreated controls. Stimulation of FAAH occurred through up-regulation of gene expression at transcriptional and translational level and was specific. Indeed, neither the activity of the anandamide-synthesizing *N*-acyltransferase and phospholipase D, nor the activity of the anandamide transporter, nor the binding to cannabinoid receptors were affected by progesterone under the same experimental conditions. The activation of FAAH by progesterone was paralleled by a decrease (down to 60%) of the cellular levels of anandamide and involved increased nuclear levels of the transcription factor Ikaros. Analysis of the FAAH promoter showed an Ikaros binding site, and mutation of this site prevented FAAH activation by progesterone in transient expression assays. Electrophoretic mobility shift and supershift assays further corroborated the promoter activity data. Furthermore, the effect of progesterone on FAAH promoter was additive to that of physiological amounts of leptin, which binds to a cAMP response element-like site in the promoter region. Taken together, these results suggest that progesterone and leptin, by up-regulating the FAAH promoter at different sites, enhance FAAH expression, thus tuning the immunomodulatory effects of anandamide. These findings might also have critical implications for human fertility.

Anandamide (arachidonoylethanolamide, AEA)[1] belongs to a group of endogenous lipids, which include amides, esters, and ethers of long chain polyunsaturated fatty acids, collectively termed "endocannabinoids" (1). It binds to cannabinoid receptors (CBR) in the central nervous system and in peripheral cells, thus having many central actions (2). Among the peripheral activities of AEA, the regulation of fertility (3) and immune function (4) has attracted growing interest. These biological actions of AEA are terminated by cellular uptake through an AEA membrane transporter (AMT) (5), followed by degradation to ethanolamine and arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH) (6). Human lymphocytes have functional CBR, AMT, and FAAH, and the latter enzyme has been shown to play a critical role in regulating human pregnancy (7). Progesterone (P) is a hormone essential for the maintenance of pregnancy and is also known to modulate immune function (8) and to elicit an immunological response critical for normal gestation (9). Indeed, P has been shown to favor the development of human T lymphocytes producing type 2 T-helper (Th2) cytokines (interleukins 4 and 10), which inhibit Th1-type cytokines (interleukin-12 and interferon-γ), thus allowing the survival of fetal allograft and therefore a successful pregnancy (10, 11). In this context, the activity of FAAH in maternal peripheral lymphocytes is under control of progesterone, which binds to its intracellular receptor and activates FAAH expression (12). As a consequence, the release of cytokines critical for fertility, such as the leukemia inhibitory factor, is favored (12). Moreover, lymphocyte FAAH has been shown to control the levels of blood AEA in pregnant women, where low FAAH activity implies high AEA levels, leading to spontaneous abortion (7, 12, 13). Taken together, these data have suggested a cross-talk between steroid hormones, cytokines, and the peripheral endocannabinoid system in lymphocytes, which is implicated in regulating immunity and fertility in humans (14). Also leptin (L), the 16-kDa non-glycosylated product of the *obese* gene, serves systemic functions, which include regulation of fertility (15) and modulation of immune response (16). Recently, L has been shown to reduce the levels of AEA in the hypotalamus of *ob*/*ob* mice, suggesting that this compound partakes of the neural circuitry regulated by L (17). In addition, L enhances FAAH expression by activating a CRE (cAMP response element)-like site in the promoter region (18).

Based on this background, we sought to investigate whether

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[1] The abbreviations used are: AEA, anandamide (*N*-arachidonoylethanolamine); 2-AG, 2-arachidonoylglycerol; AMT, AEA membrane transporter; CAT, chloramphenicol acetyltransferase; CBR, cannabinoid receptors; CP55.940, 5-(1,1′-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl)cyclohexyl]phenol; CRE, cAMP-response element; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FAAH, fatty acid amide hydrolase; GAR-AP, goat anti-rabbit antibodies conjugated with alkaline phosphatase; Ik, Ikaros; L, leptin; NAT, *N*-acyltransferase; P, progesterone; RT, reverse transcriptase; PLD, phospholipase D; NAPE, *N*-acylphosphatidylethanolamines.

P might regulate AEA metabolism in human T lymphocytes. In fact, we show an enhancement of FAAH activity and expression by P, triggered through overexpression of the Ikaros (Ik) transcription factor (19, 20) and subsequent Ik-dependent up-regulation of promoter activity. We also show that the effect of P is synergistic with that of L.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. AEA, P, mifepristone (RU486), L (human recombinant), and 1,2-dipalmitoyl-*N*-palmitoylphosphatidylethanolamine were purchased from Sigma. [^{3}H]AEA (223 Ci/mmol) and [^{3}H]CP55.940 (5-(1,1′-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl)cyclohexyl]phenol; 126 Ci/mmol) were from PerkinElmer Life Sciences. 1,2-Dioleoyl-3-phosphatidyl[2-^{14}C]ethanolamine (55 mCi/mmol) and 1,2-di[1-^{14}C]palmitoylphosphatidylcholine (111 mCi/mmol) were from Amersham Biosciences (Uppsala, Sweden). Anti-FAAH polyclonal antibodies were elicited in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR (21) conjugated to ovalbumin and were prepared by Primm S.r.l. (Milan, Italy). Rabbit anti-Ik antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA), and goat anti-rabbit antibodies conjugated to alkaline phosphatase (GAR-AP) were from Bio-Rad.

Isolation and Treatment of T Lymphocytes—Blood samples (20 ml per donor) were drawn from the antecubital vein of healthy donors (age range 28–35 years), who gave informed consent to the study, and were collected into heparinized sterile tubes. Clearance of the local Ethics Committee was obtained to sue the human cells. Peripheral lymphocytes were purified by gradient centrifugation, using the density separation medium Lymphoprep (Nycomed Pharma, Oslo, Norway), and then T-cells were isolated from the whole lymphocyte population by means of the Dynal CD2 CELLection kit (Dynal, Olso, Norway), as reported previously (12). Purified T lymphocytes were resuspended in RPMI 1640 medium (Invitrogen, Paisley, UK), supplemented with 25 mM Hepes, 2.5 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen), at a density of 1.5×10^6 cells/ml in ventilated 25-ml flasks (12). Incubation of T lymphocytes with P, alone or in the presence of different compounds, was performed at 37 °C in humidified 5% CO_2 atmosphere, at the indicated concentrations and for the indicated periods of time. Cells were treated for 1 h in serum-free medium, and then heat-inactivated fetal bovine serum was added at a final concentration of 10% (12). Controls were incubated with vehicles alone. Cell viability after each treatment was tested by trypan blue dye exclusion and was found to be higher than 90% in all cases.

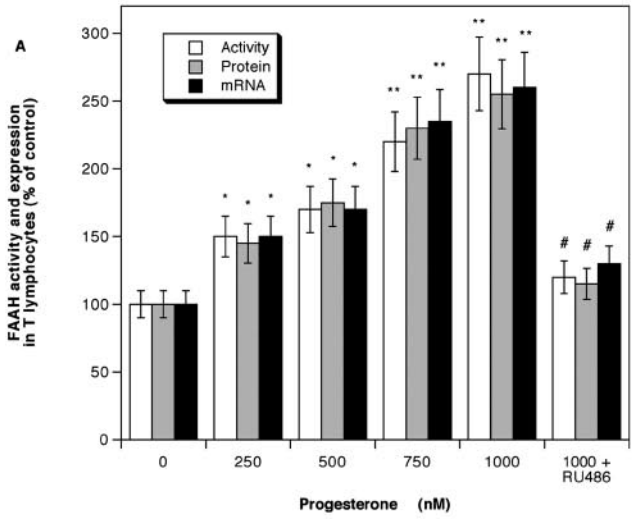
FAAH Activity and Expression—FAAH (arachidonoylethanolamide amidase; EC 3.5.1.4) activity was assayed at pH 9.0 with 10 μM [^{3}H]AEA as substrate, by the reversed phase high performance liquid chromatography method described previously (18). FAAH activity was expressed as pmol of arachidonate released per min per mg of protein. Cell homogenates (20 μg/lane) were prepared as described previously (18) and were subjected to enzyme-linked immunosorbent assay (ELISA), to quantify FAAH protein. Wells were coated with human T-cell homogenates (20 μg/well), which were then reacted with anti-FAAH polyclonal antibodies (diluted 1:300), as first antibody, and with GAR-AP, diluted 1:2000, as second antibody (12). In a previous study, the anti-FAAH antibodies have been shown to recognize a single band in human lymphocytes by Western blot analysis (12). Color development of the alkaline phosphatase reaction was measured at 405 nm, using *p*-nitrophenyl phosphate as substrate. The A_{405} 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 validated by antigen competition experiments (12). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using total RNA isolated from human T lymphocytes (10×10^6 cells) by means of the S.N.A.P.™ total RNA isolation kit (Invitrogen), as described previously (12). RT-PCR amplification of FAAH was performed using 100 ng of total RNA, and the EZ rTth RNA PCR kit (PerkinElmer Life Sciences), as reported previously (12). 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 for FAAH were as follows: (+), 5′-TGGAAGTCCTCCAAAAGCCCAG; (−), 5′-TGTCCATAGACACAGCCCTTCAG. Five μl of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, and the RT-PCR products were excised from the gel and counted in a LKB1214 Rackbeta scintillation counter (Amersham Biosciences) (12). Products were validated by size determination and sequencing, as described previously (12).

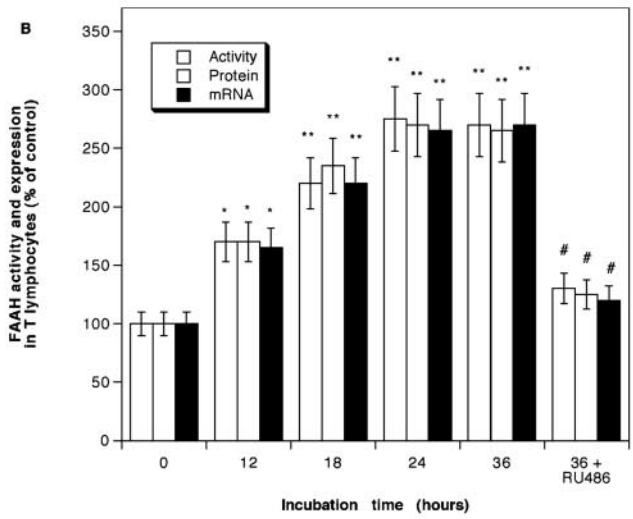
Analysis of N-Acyltransferase, Phospholipase D, Anandamide Uptake, and Cannabinoid Receptors—*N*-Acyltransferase (NAT) assay was performed as described previously (22), using 1,2-di[1-^{14}C]palmitoylphosphatidylcholine (1×10^6 dpm/test) as substrate and measuring the formation of *N*-[^{14}C]palmitoylphosphatidylethanolamines by high performance thin layer chromatography on silica gel plates (Sigma). 1,2-Dipalmitoyl-*N*-palmitoyl-phosphatidylethanolamine was used as standard, and NAT activity was expressed as pmol of *N*-palmitoylphosphatidylethanolamine formed per min per mg of protein (22). The activity of phospholipase D (PLD; EC 3.1.4.4) was assayed in T-cell homogenates by measuring the release of [^{14}C]ethanolamine from 1,2-dioleoyl-3-phosphatidyl-[2-^{14}C]ethanolamine (10 μM), as described previously (23). PLD activity was expressed as pmol of ethanolamine released per min per mg of protein. The uptake of 200 nM [^{3}H]AEA by intact T lymphocytes (2×10^6/test) through AMT was studied as described previously (18) and was expressed as pmol of AEA taken up per min per mg of protein. For CBR studies, membrane fractions prepared from T lymphocytes (10×10^6) as reported previously (18) were quickly frozen in liquid nitrogen and stored at −80 °C for no longer than 1 week. These membrane fractions were used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP55.940 (used at 400 pM), as reported previously (18). Unspecific binding was determined in the presence of 100 nM "cold" agonist (12, 18). Receptor binding was expressed as fmol of ligand bound per mg of protein.

Analysis of the Endogenous Levels of AEA and 2-Arachidonoyglycerol (2-AG)—The endogenous levels of AEA and 2-AG in human T lymphocytes (50×10^6/test) were assayed at NIAAA/National Institutes of Health by liquid chromatography/mass spectrometry, using an Agilent 1100 series LC-MSD equipped with a thermostated autosampler and column compartment, as reported previously (24). The liquid chromatography/mass spectrometry analysis was also corroborated by gas chromatography-electron impact mass spectrometry, performed in Rome as described previously (25).

Western Blot Analysis of Nuclear Levels of Ikaros—For the determination of Ik isoforms in T lymphocytes, nuclear extracts were prepared from T-cell suspensions (19), and aliquots (50 μg protein) were loaded onto 10% SDS-polyacrylamide gels and were electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad), as described previously (19). For immunodetection, anti-Ik antiserum was diluted 1:1000, and the second antibody (GAR-AP) was diluted 1:2000 (12). Protein content was normalized before loading onto the gel (18), and equal loading of extracts was verified by Ponceau staining (19). Rainbow molecular mass markers (Amersham Biosciences) were bovine serum albumin (66.0 kDa) and ovalbumin (46.0 kDa). Nuclear levels of total Ik isoforms were further quantified by ELISA, performed by coating each well with 25 μg protein/sample, as described above for FAAH, and then reacted with anti-Ik antiserum (1:1000) and GAR-AP (1:2000).

Construction of Chloramphenicol Acetyltransferase Expression Vectors and Transient Transfection—Sequence information for the upstream regulatory region of *FAAH* gene was downloaded from GeneBank™ (region: gi/11423254:644582-754250, International Human Genome Project), and the proximal promoter region of base pairs from +1 to −107 (+1 being the first nucleotide of the FAAH mRNA) was assembled using synthetic oligonucleotides (Amersham Biosciences). The DNA was gel-purified and subcloned into the *Pst*I/*Xba*I sites of pCAT3-Basic vector (Promega Corp., Madison, WI). The same strategy was used to introduce mutations in the recombinant plasmids bearing the promoter region. The nucleotide sequences of all constructs were verified by dideoxynucleotide chain termination sequencing with a Sequenase kit 2.0 (United States Biochemical, Cleveland, OH). Human T-cells (1×10^6 per test) were transfected in triplicate using TransFast™ transfection reagent (Promega Corp.), according to the manufacturer's instructions. Typically, cells were washed in phosphate-buffered saline and resuspended in 0.5 ml of serum-free medium, and then they were mixed with 0.5 ml of serum-free medium containing 2 μg of total DNA and the TransFast™ transfection reagent, at a charge ratio of 1:1 with respect to DNA. Transfection efficiency was monitored by use of 0.5 μg of thymidine kinase β-galactosidase construct (Clontech, Palo Alto, CA). After transfection, the medium was replaced with complete growth medium, and cells were harvested 48 h later. For chloramphenicol acetyltransferase (CAT) activity assays, cellular extracts were prepared as described above for FAAH, and different aliquots were used for CAT assays, for β-galactosidase activity determination, a marker of transfection efficiency, and for protein quantitation. CAT activity was determined using the Quan-T-CAT assay system (Amersham Biosciences), whereas the activity of β-galactosidase was assayed using the β-galactosidase enzyme system (Promega Corp.). The values





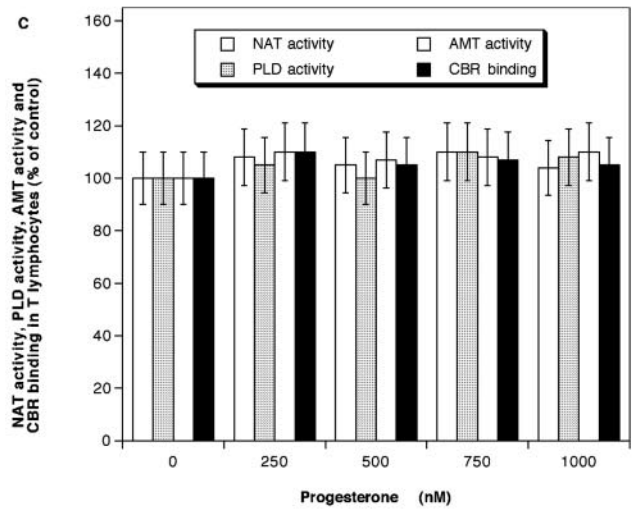


FIG. 1. **Effect of P on FAAH expression, NAT, PLD, AMT, and CBR in T-cells.** *A*, dose dependence of the activity (*white bars*), the protein content (*gray bars*), and the mRNA level (*black bars*) of FAAH in human T lymphocytes, after 24 h of incubation (100% = 140 ± 15 pmol·min^{-1}·mg protein^{-1}, for the activity, 0.220 ± 0.025 absorbance units at 405 nm, for the protein content, and 15,000 ± 1500 cpm for the mRNA level). *B*, time course of the effect of 1 μM P on FAAH activity (*white bars*), protein content (*gray bars*), and mRNA level (*black bars*) in human T-cells (100% as in *A*). *C*, activity of NAT, PLD, and AMT and CBR binding in T-cells treated for 24 h with different doses of P (100% = 10 ± 2 pmol·min^{-1}·mg protein^{-1}, for NAT activity, 130 ± 15 pmol·min^{-1}·mg protein^{-1}, for PLD activity, 50 ± 5 pmol·min^{-1}·mg protein^{-1}, for AMT activity, or 20,000 ± 2000 cpm·mg protein^{-1}, for CBR binding). In *A* and *B*, RU486 was used at 10 μM, and * denotes $p < 0.05$ *versus* control, ** denotes $p < 0.01$ *versus* control, and # denotes $p < 0.01$ *versus* 1000 nM P, in *A*, or *versus* 36 h, in *B* ($p > 0.05$ in all other cases). In all panels, *vertical bars* represent S.D. values.

TABLE I
Endogenous levels of AEA and 2-AG in human T lymphocytes

T lymphocyte treatment	Endocannabinoid level	
	AEA	2-AG
	pmol·mg protein^{-1}	
None	2.03 ± 0.20 (100%)	83.80 ± 8.20 (100%)
1 μM P for 24 h	1.23 ± 0.15[a] (60%)	55.88 ± 5.15[a] (67%)
1 μM P + 10 μM RU486 for 24 h	1.83 ± 0.20[b] (90%)	80.80±8.00[b] (96%)

[a] $p < 0.05$ *versus* control.
[b] $p < 0.05$ *versus* 1 μM P.

of CAT activity were normalized to β-galactosidase activity and to the protein content (18).

Nuclear Extracts, Electrophoretic Mobility Shift, and Supershift Assays—Nuclear extracts were prepared according to Schreiber *et al*. (26) with the modifications reported by Lee *et al*. (27). Electrophoretic mobility shift assay (EMSA) experiments were performed as described previously (27), using the Ikaros oligonucleotide −76 5′-AGGCGGGC-G**TGGGAT**CCCGGCTG-3′ −54 (site in bold), whereas the oligonucleotides used for the cold competitions were 5′-CTCGCAGCCTGGGAA-GATAAGTGG-3′ (Ikaros site derived from vasoactive intestinal peptide receptor-1 promoter), and −76 5′-AGGCGGGCGTTTTTTCCCGGCT-G-3′ −54, which is the mutated site used for the transfection experiments (the mutated nucleotides are underlined) (28). In all oligonucleotides, the numbers refer to positions in the FAAH promoter. The complexes were resolved on non-denaturing 6% polyacrylamide gels in 0.5 × TBE buffer (0.45 M Tris borate, 10 mM EDTA, pH 8.0) for 1 h at 14 V/cm and were autoradiographed overnight. For gel supershift analysis, nuclear extracts were preincubated overnight at 4 °C with 3 μg of rabbit anti-Ik antiserum, before addition of ^{32}P-labeled oligoucleotide (26). Dye was omitted from the loading buffer, and the gel was run at 4 °C in 0.2 x TBE buffer at 5 V/cm. The autoradiographic films were subjected to densitometric analysis by means of a Floor-S MultiImager equipped with Quantity One software (Bio-Rad), as reported previously (18).

Statistical Analysis—Data reported in this paper are the mean (±S.D.) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the non-parametric Mann-Whitney test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science, San Diego, CA).

RESULTS

Progesterone Stimulates FAAH Activity and Expression in Human T Lymphocytes—In vitro treatment of human T-cells with P for 24 h enhanced FAAH activity in a dose-dependent manner (Fig. 1*A*). 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. Activation of FAAH by 1 μM P was fully reverted by the synthetic antiprogestinic compound RU486 (29), used at 10 μM (Fig. 1*A*). Time course experiments showed that P-induced activation of FAAH was significant ($p < 0.05$) 12 h after T lymphocytes treatment and reached a maximum at 24 h (Fig. 1*B*). Anti-FAAH antibodies were used to quantify FAAH content in T-cells by ELISA and showed that P increased FAAH protein in human T lymphocytes in parallel to the increase of enzymic activity (Fig. 1, *A* and *B*). RT-PCR amplification of cDNA of human T lymphocytes, followed by liquid scintillation counting of RT-PCR products, showed that

P increased dose- and time-dependently also FAAH mRNA in human T lymphocytes, in a way parallel to that of enzymic activity and protein content (Fig. 1, *A* and *B*). Remarkably, treatment of T-cells with 10 μM RU486 fully prevented the increase in FAAH protein and mRNA levels induced by 1 μM P (Fig. 1, *A* and *B*). These data closely resemble our previous report on the effect of P on the whole population of peripheral lymphocytes and are in keeping with the observation that FAAH activity in T-cells accounts for 85% of the activity measured in the whole cell population (12). In addition, treatment of T lymphocytes with 1 μM P for 24 h significantly ($p < 0.05$) reduced the endogenous levels of AEA in these cells to 60% of the controls (Table I). It also reduced to a similar extent (67%) the cellular level of 2-AG, an endocannabinoid that is also cleaved by FAAH (17), and 10 μM RU486 fully reverted these effects of P (Table I).

NAT, PLD, AMT, and CBR Are Not Affected by Treatment of T Cells with Progesterone—NAT catalyzes the biosynthesis of *N*-arachidonoylphosphatidylethanolamines by transferring an arachidonate group from the *sn*-1 carbon of phospholipids to the amino group of phosphatidylethanolamines (22). Together with the *N*-acylphosphatidylethanolamines (NAPE)-hydrolyzing PLD (23), NAT is considered the checkpoint in AEA synthesis (22–25). The activity of NAT and that of PLD were almost identical in T-cells untreated or treated with 1 μM P (Fig. 1*C*). Although the activity of PLD was assayed under conditions found to be optimal for the NAPE-hydrolyzing enzyme (23), a radiolabeled phosphatidylethanolamine was used as substrate instead of radiolabeled NAPEs, which are not yet commercially available (25). To date there is no evidence that this substrate is specific for the NAPE-hydrolyzing PLD, and there are no specific inhibitors of this enzyme that might allow to further extend its analysis and to conclusively assess its contribution to AEA metabolism. However, the lack of effect of P on the activity of both NAT and PLD in T lymphocytes seems to speak in favor of a lack of effect on AEA synthesis. Also the activity of AMT was not affected by 1 μM P (Fig. 1*C*), suggesting that the cellular uptake of AEA was not coupled to its breakdown by FAAH (12, 30). In this context, it seems noteworthy that the relationship between AMT and FAAH is still under debate, because FAAH might not quite need a transporter to get in contact with AEA (31), and AMT might work "in reverse" to export (rather than import) AEA (32). Human lymphocytes have CB receptors (12) and treatment with 1 μM P for 24 h did not affect their ability to bind [^{3}H]CP55.940 through these receptors (Fig. 1*C*). Taken together with the data on FAAH, these results show for the first time that P down-regulates the tone of endocannabinoids in human T lymphocytes by activating their degradation by FAAH, rather than by reducing their synthesis through NAT and PLD or transport through AMT. To further elucidate the modulation of FAAH expression by P, we investigated the properties of the FAAH promoter in human T lymphocytes.

Analysis of the FAAH Promoter—The human *FAAH* gene has been located on chromosome 1 (33), which has been completely sequenced. Therefore, we inspected this chromosome to gain insight into the FAAH promoter features. Human FAAH promoter was found to lack TATA boxes, and like many genes bearing this feature, it had a proximally positioned SP1 site (Fig. 2*A*). Moreover, there was another SP1 site in the reverse orientation ~100 nucleotides upstream (Fig. 2*A*), a feature that resembles the structure of the mouse proximal promoter (34). P had no effect on the FAAH minimal promoter (Fig. 3, *min*), but it up-regulated the reporter *CAT* gene if under control of the region between the two SP1 sites of the FAAH upstream region (Fig. 3, *wt*). RU486 (10 μM) fully reverted the effect of 1 μM P on

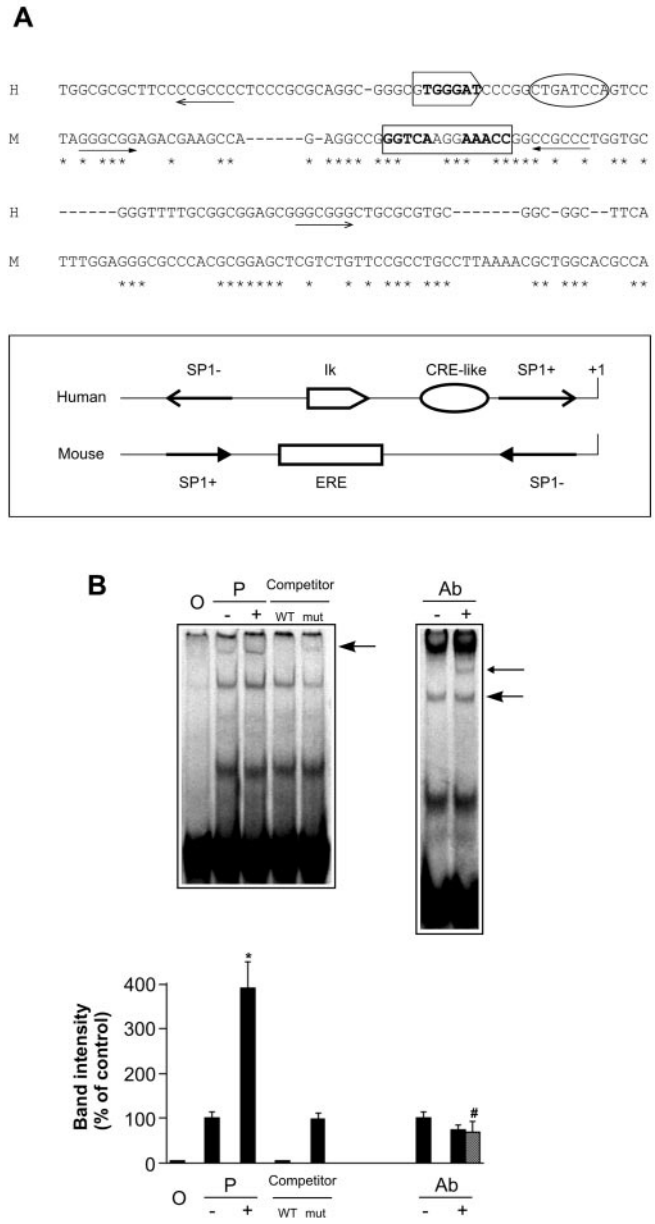


FIG. 2. **Analysis of FAAH promoter by gel shift and supershift assay.** *A*, paired proximal upstream regions of human (*H*) and mouse (*M*) *FAAH* genes. Identical nucleotides are marked with an *asterisk*. *Left-* and *right-handed arrows* denote SP1 sites in the − and + strands, respectively. *Pentagonal box*, Ikaros (*Ik*) site; *oval box*, CRE-like site; *rectangular box*, estrogen response element (*ERE*) site. *B*, gel shift and supershift (*Ab*) experiments were performed with 3 μg of T lymphocytes nuclear extracts prepared before (−) and after (+) stimulation with 1 μM P. *Lane O* represents ^{32}P-labeled oligonucleotide alone, which contained the Ikaros site; competitor wild type (*WT*) was a cold oligonucleotide containing the Ikaros site derived from vasoactive intestinal peptide receptor-1 promoter; competitor mutant (*mut*) was a cold oligonucleotide containing the mutated Ikaros site used in transfection experiments. Shifted and supershifted complexes are indicated with a *big* and a *small arrow*, respectively, and were subjected to densitometric analysis with *black* and *gray bars* representing shifted and supershifted complexes, respectively. These data are representative of at least three independent experiments. Values were normalized against P for gel shift, and against Ab for gel supershift, set to 100%. In *B*, *vertical bars* represent S.D. values, * denotes $p < 0.01$ *versus* P, and # denotes $p < 0.01$ *versus* Ab ($p > 0.05$ in all other cases).

CAT activity (Fig. 3). Moreover, under the same conditions that enhanced FAAH activity, P dose-dependently increased the nuclear amount of Ikaros isoforms Ik1, Ik2, and Ik3, as revealed by Western blot analysis (Fig. 4*A*). A further quantita-

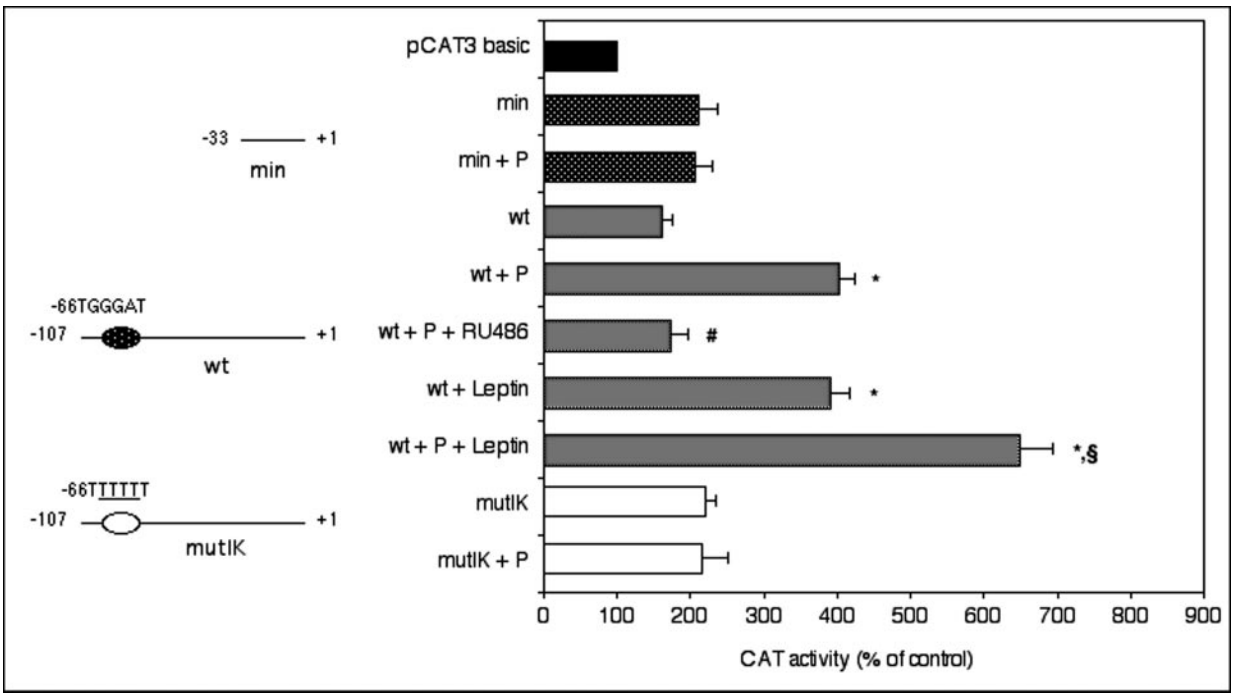


FIG. 3. **Analysis of FAAH promoter by transient expression.** 5′-Flanking regions of the human *FAAH* gene were cloned in the *Pst*I/*Xba*I sites of pCAT3-Basic vector. *min* (*black bars*), −33 to +1 upstream region containing only the proximal SP1 site; *wt* (*dark gray bars*), −107 to +1 upstream region containing the two SP1 sites flanking the Ikaros site (*black*); *mutIK* (*white bars*), same as wild type (*wt*) but with mutated Ikaros site; mutated sites and nucleotides are in *white* and *underlined*, respectively. T lymphocytes were transfected with the aforementioned constructs and left untreated or treated with 1 μM P, alone or in the presence of 10 μM RU486 or of 10 nM leptin, or treated with 10 nM leptin. Transfection efficiency was monitored by the use of thymidine kinase β-galactosidase construct. The values of CAT activity were normalized to β-galactosidase activity and to the protein content and are expressed as percent with respect to the activity of the empty vector, pCAT3-basic, which was set to 100%. * denotes $p < 0.01$ *versus* control, # denotes $p < 0.01$ *versus* wild type + P, § denotes $p < 0.05$ *versus* wild type + P ($p > 0.05$ in all other cases), and *vertical bars* represent S.D. values.

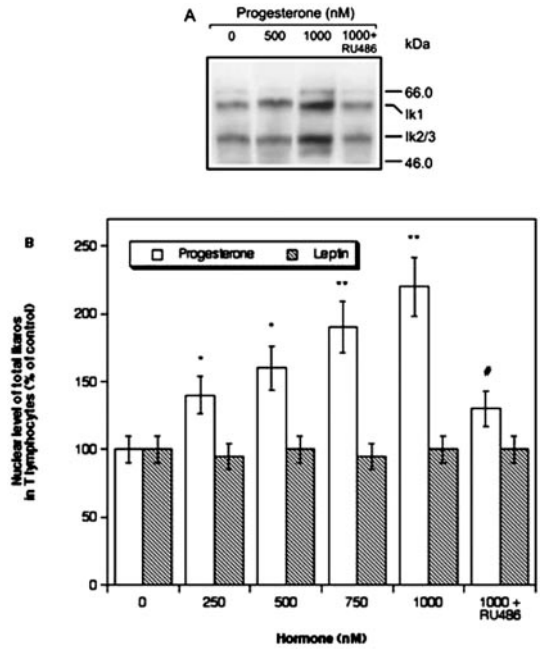


FIG. 4. **Effect of P on nuclear levels of Ikaros**. *A*, nuclear extracts (50 μg of protein) of T-cells treated for 24 h with different doses of P were analyzed by Western blot, using anti-Ikaros antiserum. Molecular mass markers and the positions of Ikaros isoforms are indicated to the *right*. These data are representative of at least three independent experiments. *B*, quantification of the nuclear levels of total Ikaros by ELISA, performed on the same samples (25 μg of proteins/well) as in *A*, or in T-cells treated for 24 h with leptin (100% = 0.180 ± 0.020 absorbance units at 405 nm). In both panels, RU486 was used at 10 μM, and * denotes $p < 0.05$ *versus* control, ** denotes $p < 0.01$ *versus* control, and # denotes $p < 0.01$ *versus* 1000 nM P ($p > 0.05$ in all other cases). *Vertical bars* represent S.D. values.

TABLE II
Modulation of FAAH expression in human T lymphocytes by P and L

T lymphocyte treatment	FAAH expression[a]		
	Activity	Protein	mRNA
	%		
None	100	100	100
1 μM P for 24 h	270 ± 25[b]	250 ± 25[b]	260 ± 25[b]
10 nM L for 24 h	330 ± 30[b]	300 ± 30[b]	310 ± 30[b]
1 μM P + 10 nM L for 24 h	570 ± 55[b,c]	530 ± 50[b,c]	550 ± 55[b,c]

[a] 100% = 140 ± 15 pmol·min^{-1}·mg protein^{-1} for the activity, 0.220 ± 0.025 absorbance units at 405 nm for the protein content, and 15,000 ± 1500 cpm for the mRNA level.
[b] $p < 0.01$ *versus* control.
[c] $p < 0.01$ *versus* 1 μM P.

tive analysis of the nuclear content of total Ik isoforms by ELISA showed that their level increased up to ~220% of the controls in the nuclei of T lymphocytes treated for 24 h with 1 μM P (Fig. 4*B*). The effect of 1 μM P was fully reverted by 10 μM RU486 (Fig. 4, *A* and *B*). On the other hand, L had no effect on nuclear Ik content under the same experimental conditions (Fig. 4*B*). Based on these data, we searched for an Ikaros consensus site in the FAAH promoter and found it at position −66 (Fig. 2*A*). Transient transfection experiments revealed that this site is indeed functional, since its mutation abolished FAAH up-regulation by P (Fig. 3, *mutIk*). Moreover, EMSA experiments showed that T-cell nuclear extracts formed a retarded complex in untreated cells which was more abundant in cells treated for 24 h with 1 μM P, when an oligonucleotide corresponding to the Ik site found at position −66 of the FAAH promoter was used (Fig. 2*B*). Specificity of the binding to the Ikaros site was confirmed by using a cold competitor, which corresponds to an established functional Ik site derived from the vasoactive intestinal peptide receptor-1 promoter (28), and

the mutated site used for transient transfection experiments. Cold competitor and mutated site, respectively, abolished or had no effect on the retarded complex (Fig. 2*B*). The complex was further retarded by using antibodies directed against the Ik isoforms in gel supershift assays, thus confirming the identity of the protein binding to the Ik site (Fig. 2*B*). Densitometric analysis of gel shift and supershift experiments strengthened the hypothesis that Ik was directly involved in the regulation of FAAH expression (Fig. 2*B*). In addition, transient expression experiments showed that P can activate FAAH promoter in synergism with L, indeed a combination of 1 μM P and 10 nM L increased CAT activity up to ~650% of the controls, whereas they increased CAT up to ~400% when used alone (Fig. 3). In this context, it should be recalled that 10 nM L has been recently shown to activate the FAAH promoter in T-cells through a CRE-like site (18), which was located downstream of the Ik site in the promoter region (Fig. 2*A*). Consistent with these data, treatment of human lymphocytes for 24 h with 1 μM P + 10 nM L increased FAAH activity, protein content, and mRNA level up to ~570, ~530, and ~550% of the untreated controls, respectively, demonstrating that L further potentiated the effect of P on FAAH expression (Table II).

DISCUSSION

In this study we show that P stimulates FAAH activity and expression in human T lymphocytes, by increasing the nuclear levels of Ikaros, and hence its binding to the FAAH promoter. In addition, we show that P can activate FAAH in synergism with L, which up-regulates a CRE-like element downstream of the Ik site. These data open new avenues for the management of immune and fertility defects under progesterone and leptin control in humans.

P stimulates FAAH activity and expression in T-cells at the same circulating levels (from 0.02 to 0.30 μg/ml) found in pregnant women (10). These data extend to T lymphocytes our previous observation that P, in the same concentration range used here, up-regulates FAAH gene expression in the whole population of peripheral lymphocytes, by binding to its intracellular receptor (12). In the same line, RU486, a synthetic antiprogestinic which stabilizes progesterone receptor in a form unable to bind DNA (29), fully reverts the effect of P on FAAH (Fig. 1, *A* and *B*). Remarkably, RU486 was effective at a concentration (10 μM) known to modulate immuno-endocrine interactions in early pregnancy of humans (35). On the other hand, P was ineffective on NAT, PLD, and AMT activity, and on CBR binding in T lymphocytes (Fig. 1*C*), suggesting that FAAH was the only checkpoint for the effect of this hormone. These observations are consistent with a role for FAAH in modulating immunoendocrine interactions in early pregnancy in humans (7, 12). They are also in keeping with the hypothesis that FAAH is the key-regulator of AEA levels *in vivo*; indeed, FAAH knock-out mice show ~15-fold higher levels of AEA than wild-type littermates (36), and AEA levels in human blood inversely correlate with FAAH activity in peripheral lymphocytes (13). In this line, we show that 1 μM P, which increased FAAH in T lymphocytes up to ~270% over controls (Fig. 1*A*), reduced AEA levels in these cells down to 60% (Table I). Interestingly, FAAH has been shown to be critically linked to drug/alcohol abuse and dependence in humans (37), again suggesting that this enzyme is pivotal for controlling the biological activity of AEA and potentially that of other FAAH-hydrolyzable congeners like 2-AG (17). At any rate, the up-regulation of FAAH expression by P in human T-cells is a major finding of this investigation, associated with higher FAAH activity and lower AEA content in these cells.

The mechanism of FAAH activation by P was further investigated by analyzing the FAAH promoter. Like many promoters lacking a TATA box, it had a proximally positioned SP1 site, which was preceded, both in the human and mouse promoters, by another SP1 site in reverse orientation (Fig 2*A*). FAAH promoter also revealed a binding site for Ikaros, a member of the Kruppel family of "zinc finger" DNA-binding proteins, which acts as a critical transcriptional regulator of lymphocyte ontogeny and differentiation (20). Ikaros comprises eight different isoforms, Ik1 through Ik3 localizing to the nucleus and Ik4 through Ik8 localizing to the cytosol (20). Recently Ik1, Ik2, and Ik3, the only isoforms that exhibit high DNA binding affinity (20), have been detected in the nucleus of human peripheral blood mononuclear cells (19, 20), where they are essential in controlling the activation of granzyme B promoter by dexamethasone (19). Interestingly, we found that human T-cells express nuclear Ik1, Ik2, and Ik3 (Fig. 4*A*) and that the same doses of P that enhanced FAAH also increased up to ~220% over the controls the nuclear levels of these Ikaros isoforms (Fig. 4*B*). Homo- and heterodimer formation between Ik1, Ik2, and Ik3 is known to greatly increase their affinity for DNA and their consequent ability to activate transcription (19, 20). Therefore, it can be concluded that P activates FAAH in human T lymphocytes, by stimulating the binding of Ikaros to DNA, thus enhancing its transcriptional activity on the FAAH promoter. Transfection experiments using FAAH promoter constructs with mutated Ikaros (mutIk) revealed that indeed this site confers P responsiveness (Fig. 3), and EMSA and gel supershift analysis further corroborated this conclusion (Fig. 2*B*). In addition, we show unprecedented evidence that the effect of P on FAAH promoter may be synergistic with that of physiological concentrations of L (Fig. 3), which up-regulates a CRE-like element downstream of the Ikaros site in the promoter region (Fig. 2*A*).

It seems noteworthy that to date only one report has described the modulation of human FAAH promoter by L (18). Two other interesting reports have characterized the promoter (38) and the transcriptional regulation (34) of mouse FAAH in neuronal cell lines. In particular, they have shown either putative (38) or imperfect (34) estrogen response elements in the FAAH promoter region, giving some ground to our previous report that estrogen down-regulates FAAH activity in mouse (39). Here, we show that in the human FAAH promoter estrogen response elements are replaced by an Ikaros binding site (Fig. 2*A*). In this context, we have recently shown that estrogen down-regulates FAAH in human cells according to a non-genomic mechanism (32). These observations suggest a relevant species specificity of FAAH regulation, although the human and mouse FAAH (localized on chromosomes 1 and 4, respectively) share 84% sequence identity (21) and have a conserved genomic structure (33). In addition, also a tissue specificity of FAAH promoter activity has been observed (38), which might further contribute to divergent regulation in different species or in different tissues of the same species. The interaction between different transcription factors, some of which have been identified here for the first time, on FAAH regulation awaits for further clarification.

In conclusion, the results reported here represent the first evidence of a link between the hormone-cytokine networks controlled in T lymphocytes by progesterone (11) and/or leptin (40), and the peripheral endocannabinoid system, and suggest that AEA hydrolysis by FAAH might be the target for new therapies of human defects in immunity and fertility.

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Progesterone Activates Fatty Acid Amide Hydrolase (FAAH) Promoter in Human T Lymphocytes through the Transcription Factor Ikaros: EVIDENCE FOR A SYNERGISTIC EFFECT OF LEPTIN

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