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Leptin Activates the Anandamide Hydrolase Promoter in Human T Lymphocytes through STAT3*

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Physiological concentrations of leptin stimulate the activity of the endocannabinoid-degrading enzyme anandamide hydrolase (fatty acid amide hydrolase, FAAH) in human T lymphocytes up to ~300% over the untreated controls. Stimulation of FAAH occurred through up-regulation of gene expression at transcriptional and translational levels and involved binding of leptin to its receptor with an apparent dissociation constant (K_d) of 1.95 ± 0.14 nM and maximum binding (B_{max}) of 392 ± 8 fmol·mg protein^{-1}. Leptin binding to the receptor triggered activation of STAT3 but not STAT1 or STAT5 or the mitogen-activated protein kinases p38, p42, and p44. Peripheral lymphocytes of leptin knockout (*ob/ob*) mice showed decreased FAAH activity and expression (~25% of the wild-type littermates), which were reversed to control levels by exogenous leptin. Analysis of the FAAH promoter showed a cAMP-response element-like site, which is a transcriptional target of STAT3. Consistently, mutation of this site prevented FAAH activation by leptin in transient expression assays. Electrophoretic mobility shift and supershift assays further corroborated the promoter activity data. Taken together, these results suggest that leptin, by up-regulating the FAAH promoter through STAT3, enhances FAAH expression, thus tuning the immunomodulatory effects of anandamide. These findings might also have critical implications for human fertility.

Leptin (L)[1] is the 16-kDa non-glycosylated product of the *obese* gene, which is secreted by adipose cells, is released into the circulation, and transported across the blood-brain barrier into the central nervous system where it regulates energy homeostasis (1). Leptin also serves systemic functions apart from those related to food intake and energy expenditure in mammals, including regulation of fertility (2) and modulation of immune response (3). These two actions might be interconnected in humans because leptin alters the production from T lymphocytes of T helper 1 and 2 cytokines (4), which are critical in regulating embryo implantation and materno-fetal exchanges (5, 6). In this line, mice genetically defective in leptin (*ob/ob* knock-out) are obese, infertile, and immunodeficient, and administration of exogenous leptin can reverse these defects (1–4). Leptin signaling is mediated by the leptin receptor (LR), which exists in at least six different isoforms (1). Yet, only the long LR isoform has all intracellular motifs necessary for signaling via the signal transducer, activator of transcription (STAT), and/or the mitogen-activated protein kinase (MAPK) pathways (7–11). The relative importance of these divergent signaling events in leptin action is still unknown. Recently, leptin has been shown to reduce the levels of anandamide (arachidonoylethanolamide, AEA) in the hypothalamus of *ob/ob* mice, suggesting that this compound partakes of the neural circuitry regulated by leptin (12).

AEA belongs to a group of endogenous lipids, which include amides, esters, and ethers of long chain polyunsaturated fatty acids, collectively termed "endocannabinoids" (13). It binds to cannabinoid receptors (CBR) in the central nervous system and in peripheral immune cells, thus having many central actions (14). Together with the congeners 2-arachidonoylglycerol (12, 15) and oleoylethanolamide (16), AEA has been implicated in the regulation of appetite. Among the peripheral activities of AEA, the regulation of fertility (17) and immune function (18) has attracted growing interest. These biological actions of AEA are terminated by cellular uptake through an AEA membrane transporter (AMT) (19), followed by degradation to ethanolamine and arachidonic acid by the enzyme AEA hydrolase (fatty acid amide hydrolase, FAAH) (20). Human lymphocytes have functional CBR, AMT, and FAAH, and the latter enzyme has been shown to play a critical role in regulating human pregnancy (21). Indeed, the expression of lymphocyte FAAH is under control of progesterone and contributes to the release of cytokines critical for fertility, such as the leukemia inhibitory factor (22). 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 (21–23). 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 (24). Therefore, we sought to investigate whether leptin might regulate AEA metabolism in human T-cells, thus assuming that the actions of this endocannabinoid on fertility and immunity could be part of the molecular events responsible for the effects of leptin. In fact, we show an enhancement of FAAH activity

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[1] The abbreviations used are: L, leptin; LR, leptin receptor; AEA, anandamide (arachidonoylethanolamide); AMT, AEA membrane transporter; CAT, chloramphenicol acetyltransferase; CBR, cannabinoid receptors; CP55.940, 5-(1,1′-dimethylheptyl)-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/M-AP, goat anti-rabbit/mouse antibodies conjugated with alkaline phosphatase; IGF-IR, insulin-like growth factor I receptor; sLR, soluble leptin receptor; MAPK, mitogen-activated protein kinase; RAG-AP, rabbit anti-goat antibodies conjugated with alkaline phosphatase; RT, reverse transcriptase; STAT, signal transducer and activator of transcription.

and expression by leptin, triggered through binding to LR and subsequent STAT3-dependent up-regulation of promoter activity.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. Leptin (human recombinant for T-cell studies, mouse recombinant for *ob*/*ob* mice injections) and anandamide (AEA) were purchased from Sigma. PD98059 and SB203580 were from Calbiochem (La Jolla, CA). [^{3}H]AEA (223 Ci/mmol), ^{125}I-labeled leptin (2200 Ci/mmol) and [^{3}H]CP55.940 (5-(1,1′-dimethylheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl) cyclohexyl]-phenol; 126 Ci/mmol) were from PerkinElmer Life Sciences. Anti-FAAH polyclonal antibodies were elicited in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR (25) conjugated to ovalbumin and were prepared by Primm S.r.l. (Milan, Italy). Mouse monoclonal antibodies against actin, STAT1, STAT3, p38 MAPK, and their phosphorylated (activated) forms, monoclonal antibodies against phosphorylated p42 and p44 MAPK, rabbit polyclonal antibodies against p42 and p44 MAPK or against STAT5, goat polyclonal antibodies against phospho-STAT5, and rabbit anti-goat antibodies conjugated to alkaline phosphatase (RAG-AP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human leptin and anti-human insulin-like growth factor I receptor (anti-IGF-IR) monoclonal antibodies, and human leptin receptor/Fc chimeras (soluble leptin receptor, sLR) were purchased from R&D Systems (Minneapolis, MN). According to the manufacturer's instructions, ~100 ng/ml of anti-leptin antibodies or of sLR are enough to neutralize the effects of 1 nM (~15 ng/ml) leptin, and in this study we used a 2-fold excess of each neutralizing agent. Goat anti-rabbit and goat anti-mouse antibodies conjugated to alkaline phosphatase (GAR-AP and GAM-AP) were from Bio-Rad (Hercules, CA).

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 use 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, Oslo, Norway) as reported (22). Purified T lymphocytes were resuspended in RPMI 1640 medium (Invitrogen), 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 (22). Incubation of T lymphocytes with leptin 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. 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. Peripheral lymphocytes were isolated by means of Lymphoprep (Nycomed Pharma) also from leptin knock-out (*ob*/*ob*) mice and their wild-type littermates, purchased from Jackson Laboratories (Bar Harbor, ME). Wild-type and *ob*/*ob* mice (6 per group) received a single intravenous injection of 250 μg of mouse recombinant leptin, or vehicle in the controls, and were sacrificed 24 h later by decapitation (12). Blood was immediately collected and peripheral lymphocytes were isolated. All animal experimental protocols were approved by the local Committee on Animal Care and Use and met the guidelines of the National Institutes of Health, detailed in the *Guide for the Care and Use of Laboratory Animals*, and of the European Community directives regulating animal research.

Anandamide Hydrolase Activity and Expression—Fatty acid amide hydrolase (EC 3.5.1.4; FAAH) activity was assayed at pH 9.0 with 10 μM [^{3}H]AEA as substrate by the reversed phase high performance liquid chromatography method already described (26). Cell homogenates (20 μg/lane) were prepared as described (26) and were subjected to SDS-PAGE (12%) under reducing conditions. Rainbow molecular mass markers (Amersham Biosciences) were phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (46.0 kDa) and soybean trypsin inhibitor (27.0 kDa). For immunochemical analysis, gels were electroblotted onto 0.45 μm nitrocellulose filters (Bio-Rad), and FAAH was visualized with anti-FAAH polyclonal antibodies (1:200), using GAR-AP diluted 1:2000 as second antibody (22). Actin was immunodetected with anti-actin monoclonal antibodies (1:500), using GAM-AP diluted 1:2000 as second antibody (22). Densitometric analysis of filters was performed by means of a Floor-S MultiImager equipped with a Quantity One software (Bio-Rad). The same anti-FAAH antibodies were used to further quantify FAAH protein by enzyme-linked immunosorbent assay (ELISA). Wells were coated with human T-cell or mouse peripheral lymphocyte 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 (22). 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 (22). Reverse transcriptase (RT)-PCR was performed using total RNA isolated from human T lymphocytes (10×10^6 cells) by means of the SNAP™ Total RNA Isolation Kit (Invitrogen, Carlsbad, CA) as described (22). RT-PCR reactions were performed using 100 ng of total RNA for the amplification of FAAH or 0.4 ng for 18 S rRNA and the EZ rTth RNA PCR kit (PerkinElmer Life Sciences). 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′-TGGAAGTCCTCCAAAAGCCCAG, (-) 5′-TGTCCATAGACACAGCCC-TTCAG, for FAAH; (+) 5′-AGTT-GCTGCAGTTAAAAAGC, (-) 5′-CCTCAGTTCCGAAAACCAAC for 18 S rRNA.

Five μl of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography (22). The autoradiographic films were subjected to densitometric analysis by means of a Floor-S MultiImager 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 (Amersham Biosciences). Products were validated by size determination and sequencing as described (22).

Analysis of Anandamide Uptake, Cannabinoid Receptor, and Leptin Receptor—The uptake of 200 nM [^{3}H]AEA by intact T lymphocytes (2×10^6/test) through the AMT was studied as described (26). For CBR studies, membrane fractions were prepared from T lymphocytes (10×10^6) as reported (26), 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 (400 pM) as described previously (26). The same filtration assays were used to analyze the binding of ^{125}I-labeled leptin to T-cells (9, 27). In this case, apparent dissociation constant (K_d) and maximum binding (B_{max}) values were calculated from saturation curves in the range 0–15 nM, elaborating the binding data through nonlinear regression analysis with the Prism 3 program (GraphPAD Software for Science, San Diego, CA) (26). Unspecific binding was determined in the presence of 100 nM "cold" leptin (9, 27).

Western Blot Analysis of Protein Phosphorylation— For the analysis of total STAT1, STAT3, and STAT5 of p38, p42, and p44 MAPK and of the corresponding phosphorylated (activated) forms, whole cell extracts were prepared as reported (11). Cell lysates (50 μg of protein) were loaded onto 10% SDS-polyacrylamide gels and were then electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad) as described above for FAAH. For immunodetection, the specific first antibody was diluted 1:1000, and the appropriate second antibody (GAM-AP, GAR-AP, or RAG-AP) was diluted 1:2000 (22). Protein content was normalized before loading onto the gel, and equal loading of extracts was verified by Ponceau staining (22).

Construction of Chloramphenicol Acetyltransferase (CAT) Expression Vectors and Transient Transfection—Sequence information for the upstream regulatory region of the *FAAH* gene was downloaded from GenBank™ (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 Corporation, 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 (USB, Cleveland, OH). Human T-cells (1×10^6 per test) were transfected in triplicate using TransFast™ Transfection Reagent (Promega) 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

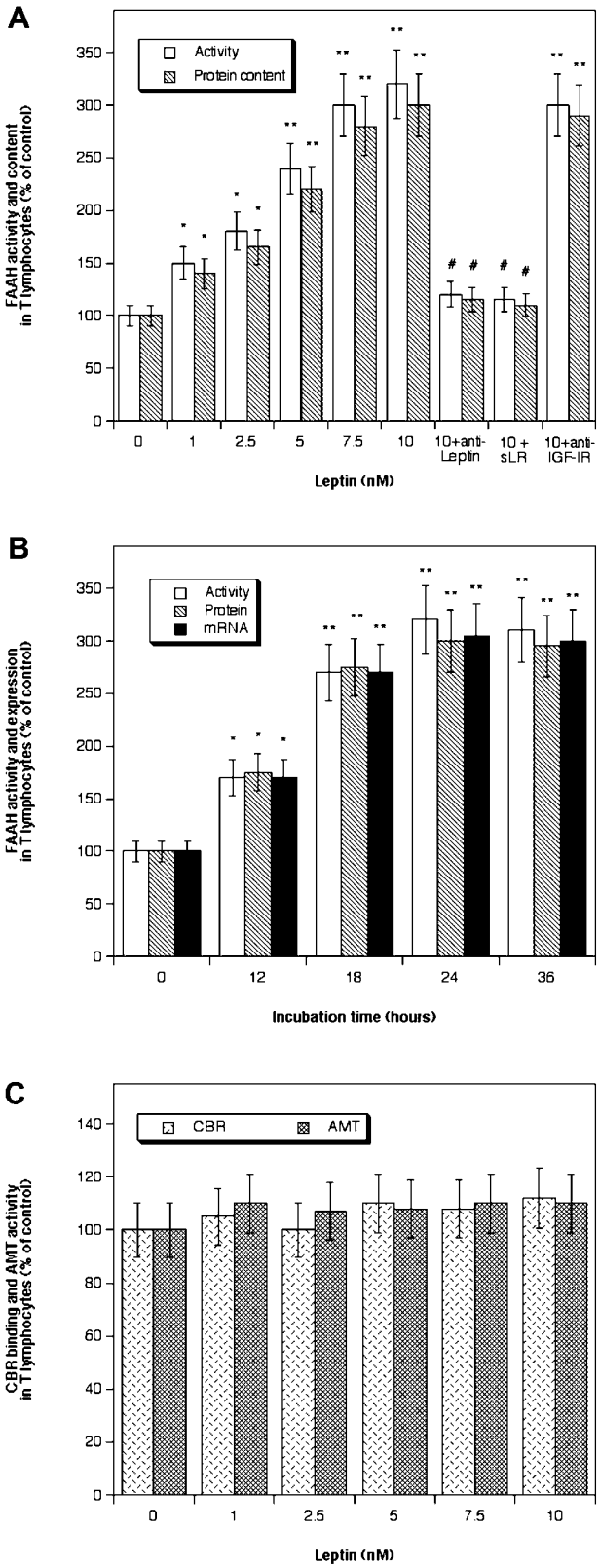


FIG. 1. **Effect of leptin on FAAH activity, CBR binding, and AMT activity.** *A*, effect of leptin on the activity (*white bars*) and the protein content (*hatched bars*) of FAAH. Human T lymphocytes were incubated for 24 h with leptin alone or with 10 nM (~150 ng/ml) leptin in the presence of 3 μg/ml anti-leptin antibodies, sLR, or anti-IGF-IR mock antibodies (100% = 140 ± 15 pmol·min^{-1}·mg protein^{-1} for the activity or 0.220 ± 0.025 absorbance units at 405 nm for the protein content). *B*, time-course of the effect of 10 nM leptin on FAAH activity (*white bars*), protein content (*hatched bars*), and mRNA level (*black bars*) in human T-cells (100% as in *A* for the activity and the protein content; 100% = 15000 ± 1500 cpm for the mRNA level). *C*, CBR binding and AMT activity in T-cells treated for 24 h with 10 nM leptin (100% = 20000 ± 2000 cpm·mg protein^{-1} for CBR binding or 50 ± 5 pmol·min^{-1}·mg protein^{-1} for AMT activity). In *A–B*, * denotes $p < 0.05$ *versus* control and ** denotes $p < 0.01$ *versus* control. In all panels, *vertical bars* represent S.D. values.

transfection, the medium was replaced with complete growth medium, and cells were harvested 48 h later. For 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). The values of CAT activity were normalized to β-galactosidase activity and to the protein content, and the relative CAT values were the average of at least three independent experiments, each performed in duplicate.

Nuclear Extracts, EMSA, and Gel Supershift Assays—Nuclear extracts were prepared according to Schreiber and co-workers (28) with the modifications reported by Lee and co-workers (29). EMSA experiments were performed as described (29), deriving the sequence for the wild-type cAMP responsive element (CRE)-like site bandshift from the FAAH promoter region: −61 5′-CCCGGC**TGATCCA**GTCCG-3′ −44 (site in *bold*). The sequence for the mutated site was the same used for the transfection experiments, *i.e.* −61 5′-CCCGGCAAATCAAAGTCCG-3′ −44 (mutated nucleotides are *underlined*). The numbers in the oligonucleotides refer to positions in the FAAH promoter. The complexes were resolved on non-denaturing 6% polyacrylamide gels in 0.5 x TBE buffer 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 a mixture of anti-STAT3 polyclonal antibodies, consisting of equimolar amounts of sc-482X, sc-483X, sc-7993X, and sc-8001X (Santa Cruz Biotechnology) before addition of ^{32}P-labeled oligonucleotide (31). Dye was omitted from the loading buffer, and the gel was run at 4 °C in 0.2× TBE buffer at 5 V/cm.

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).

RESULTS

Leptin Stimulates FAAH Activity and Expression in Human T Lymphocytes—In vitro treatment of human T-cells with L for 24 h enhanced FAAH activity in a dose-dependent manner (Fig. 1*A*). FAAH activation reached statistical significance ($p < 0.05$) at 1 nM L and a maximum at 10 nM. Therefore, the last concentration was chosen to further investigate the effect of L on FAAH. FAAH activation by 10 nM L (corresponding to ~150 ng/ml) was fully prevented by anti-leptin antibodies or by sLR, both used at neutralizing concentrations of 3 μg/ml. Instead, "mock" antibodies against IGF-IR were ineffective at the same concentration (Fig. 1*A*). Time-course experiments showed that L-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*). Western blot analysis of T lymphocyte extracts showed that specific anti-FAAH antibodies recognized a single immunoreactive band of the molecular size expected for FAAH, the intensity of which was dose dependently higher in L-treated than in control cells (Fig. 2*A*). Densitometric analysis of the filter shown in Fig. 2*A* (representative of triplicate experiments) indicated that FAAH protein increased to 135, 175, and 235% of the control (100% = 11500 ± 1200 units/mm^2) in cells treated for 24 h with 1, 5, or 10 nM L, respectively. On the other hand, T-cells expressed the same levels of actin at all concentrations of leptin (Fig. 2*A*), ruling out that the different levels of FAAH in these cells might be due to unequal loading of proteins. The same anti-FAAH antibodies were used to further quantify FAAH content by ELISA, which showed that L increased FAAH protein in human T lymphocytes in parallel to the increase of enzymic activity (Fig. 1, *A* and *B*). RT-PCR

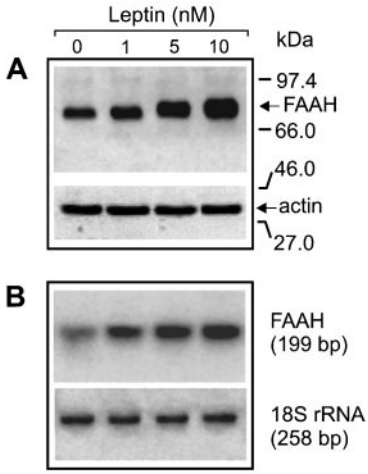


FIG. 2. **Effect of leptin on FAAH expression.** *A*, Western blot analysis of human lymphocytes, treated with different amounts of leptin and reacted with specific anti-FAAH (*upper panel*) or anti-actin (*lower panel*) antibodies. Molecular mass markers and the positions of FAAH and actin are indicated to the *right*. *B*, RT-PCR analysis of cDNA of the same samples as in *A*. The expected sizes of the amplicons (199 bp for FAAH and 258 bp for 18 S rRNA) are indicated to the *right*. These data are representative of three independent experiments.

amplification of cDNA of human T lymphocytes showed a single band of the expected molecular size for *FAAH* gene, which increased dose dependently in L-treated cells (Fig. 2*B*). Densitometric analysis of the autoradiographic film shown in Fig. 2*B* (representative of triplicate experiments) indicated that FAAH mRNA increased to 120, 165, and 200% of the control (100% = 2500 ± 280 units/mm^2) in cells treated for 24 h with 1, 5, or 10 nM L, respectively. Under the same experimental conditions, the expression of the 18 S rRNA gene was unaffected (Fig. 2*B*). Liquid scintillation counting of RT-PCR products showed that L increased time dependently FAAH mRNA in human T lymphocytes in a way parallel to that of enzymic activity and protein content (Fig. 1*B*). Remarkably, treatment of human lymphocytes with 10 nM leptin for 24 h reduced the levels of AEA in these cells from ~3.0 to ~0.5 pmol/10^8 cells.[2] On the other hand, the same concentrations of leptin, which enhanced FAAH activity, and expression were ineffective on CBR binding and AMT activity in T-cells (Fig. 1*C*). Furthermore, FAAH activity and protein content in peripheral lymphocytes from leptin knock-out (*ob*/*ob*) mice were ~25% of the levels in wild-type controls (Table I). Intravenous injection of a single dose of mouse recombinant leptin (250 μg/ml) into *ob*/*ob* mice was able to increase FAAH activity and content in peripheral lymphocytes to control levels, whereas it was ineffective on FAAH of wild-type mice (Table I).

Human T Lymphocytes Have a Functional Leptin Receptor—Human T-cells were able to bind ^{125}I-labeled leptin according to a saturable process (Fig. 3*A*), which yielded an apparent dissociation constant (K_d) of 1.95 ± 0.14 nM and maximum binding (B_{max}) of 392 ± 8 fmol·mg protein^{-1}. These values are in agreement with previous reports on LR of human hepatic cells (7, 30) and on LR stably transfected into different cell types (7, 27). Cold leptin fully displaced ^{125}I-labeled leptin from its binding site when used at 100 nM, whereas AEA was ineffective at concentrations up to 10 μM (Fig. 3*A*).

Activation of Downstream Signals by Binding of Leptin to LR in T-cells—The binding of leptin to the long form of its receptor is known to activate different signaling pathways by phosphorylating either STAT 1, 3, or 5 or p42/p44 MAPK (8, 9, 31). Also activation of p38 MAPK (10) can be part of the signaling cascade triggered by LR. Therefore, the levels of these proteins were measured in L-treated T lymphocytes. The non-phosphorylated, inactive forms of each of the signals studied were present in T-cells, yet only phosphorylated STAT3 increased

[2] M. Maccarrone, J. Harvey-White, G. Kunos, and A. Finazzi-Agrò, unpublished results.

TABLE I
FAAH activity and expression in peripheral lymphocytes isolated from wild-type and leptin knock-out (ob/ob) mice

The activity and expression of FAAH in mouse peripheral lymphocytes was determined 24 h after intravenous injection of 250 μg of leptin (or vehicle in the controls) and was expressed as percentage of the untreated controls (100% = 150 ± 15 pmol·min^{-1}·mg protein^{-1} for the activity and 0.220 ± 0.025 A_{405} units for the protein content).

Mouse strain	FAAH	
	Activity	Protein content
	% of control	
Wild-type	100	100
Ob/ob	22 ± 3[a]	25 ± 3[a]
Wild-type + leptin	110 ± 10	105 ± 10
Ob/ob + leptin	85 ± 9[b]	90 ± 9[b]

[a] Denotes $p < 0.01$ *versus* wild-type;
[b] denotes $p < 0.01$ *versus ob/ob* ($n = 6$ for each group).

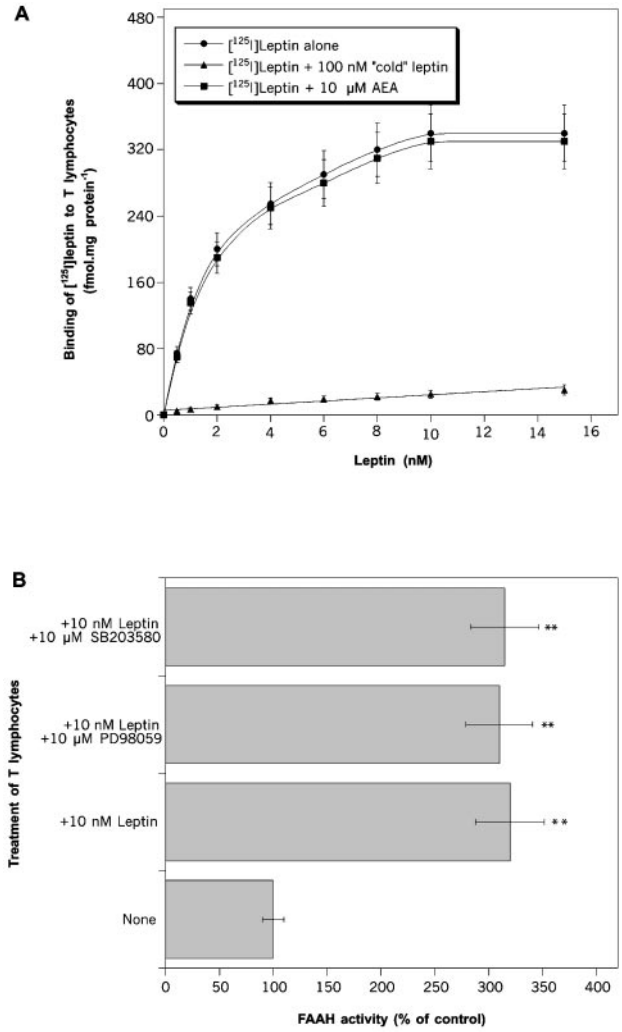


FIG. 3. **Characterization of LR.** *A*, saturation curves of the binding of ^{125}I-labeled leptin to human T lymphocytes alone (*circles*) or in the presence of 100 nM cold leptin (*triangles*) or of 10 μM anandamide (*squares*). *B*, effect of p42/44 MAPK inhibitor PD98059 and of p38 MAPK inhibitor SB203580 (each used at 10 μM) on the activation of FAAH induced by a 24-h treatment of T-cells with 10 nM leptin. In *B*, ** denotes $p < 0.01$ *versus* control. In both panels, *vertical bars* represent S.D. values.

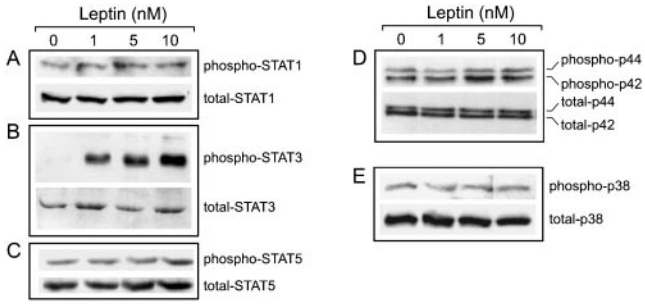


FIG. 4. **Activation of downstream signals by leptin.** Human T lymphocytes were treated for 24 h with different concentrations of leptin, and then lysates (50 μg of proteins) were immunoblotted with the specific antibody against the inactive (*total*) or active (phosphorylated, *phospho*) forms of STAT1 (*A*), STAT3 (*B*), STAT5 (*C*), p42/p44 MAPK (*D*), or p38 MAPK (*E*). The positions of phosphoproteins are indicated to the *right*. These data are representative of three independent experiments.

dose dependently in L-treated T lymphocytes (Fig. 4*B*). Densitometric analysis of filters like that shown in Fig. 4 (representative of triplicate experiments) showed that phosphorylated STAT3 was ~1.5-, ~2.4-, and ~3.5-fold higher than total STAT3 in T lymphocytes treated for 24 h with 1, 5, or 10 nM L, respectively. On the other hand, none of the other signaling proteins was activated by L under the same experimental conditions (Fig. 4, *A*, *C–E*). Consistently, the selective inhibitor of p42/p44 MAPK PD98059 (32) or the selective inhibitor of p38 MAPK SB203580 (33) were ineffective on the activation of FAAH induced in T-cells by 10 nM L (Fig. 3*B*) when used at concentrations known to inhibit the target enzyme. The lack of inhibitors of STAT1, 3, or 5 did not allow to further extend the pharmacological experiments to these proteins.

Analysis of the FAAH Promoter—The human *FAAH* gene has been located on chromosome 1 (34), which has been completely sequenced. Therefore, we inspected this chromosome to gain insight on 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. 5*A*). Moreover, there was another SP1 site in the reverse orientation ~100 nucleotides upstream (Fig. 5*A*), a feature that resembles the structure of the mouse proximal promoter (35). Inspection of the promoter sequence did not show any obvious binding site for STAT3; however, a new mechanism of transcriptional regulation based on STAT3 tethering to a CRE-like site in the LAP/C/EBPβ promoter has been recently reported (31). Such a CRE-like site was indeed present in the FAAH promoter (Fig. 5*A*), and transient transfections using constructs containing only the proximal SP1 site (−33 to +1) or both the SP1 and the CRE-like (−107 to +1) sites driving the CAT reporter gene in T lymphocytes showed that the −33 construct worked as an unregulated promoter (Fig. 6, *min*), while the −107 construct was up-regulated by leptin (Fig. 6, *wt*). The two SP1 sites in the human FAAH promoter flank a sequence that resembles a CRE-like site similar to that found in the LAP/C/EBPβ promoter (31). Disruption of this site by mutation abolished the leptin up-regulation (Fig. 6, *mutL* and *mutL + leptin*). To confirm that leptin acts through this sequence, EMSA experiments were performed using nuclear extracts prepared from T lymphocytes untreated or treated for 24 h with 10 nM leptin. As shown in Fig. 5*B*, complex formation was found only with oligonucleotides containing the CRE-like site of the FAAH promoter. Complex formation was not seen when the mutant oligonucleotide (bearing the same mutation used for the transient transfection experiment) was used as a ^{32}P-labeled probe. Gel supershift assays showed the presence of the STAT3 protein in the shifted complex (Fig. 5*B*).

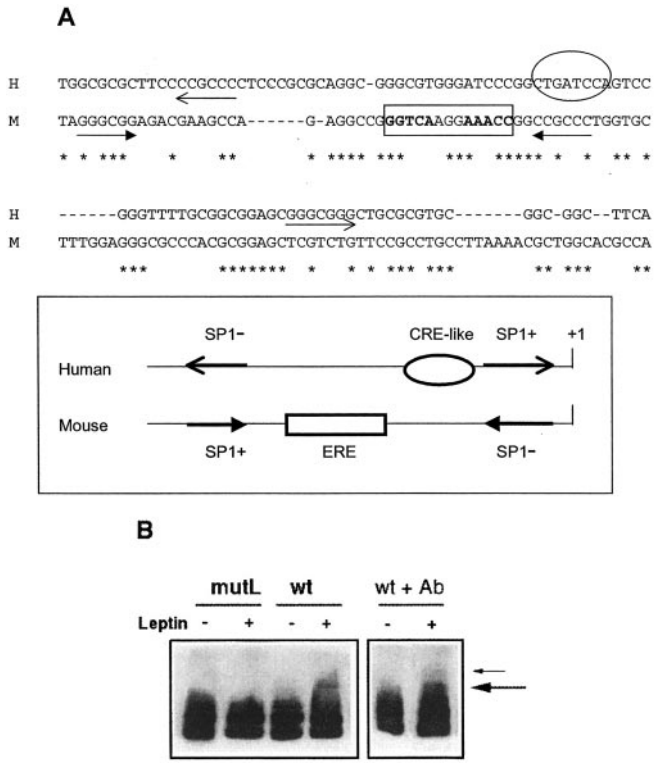


FIG. 5. **Analysis of FAAH promoter by EMSA.** *A*, paired proximal upstream regions of human (*H*) and mouse (*M*) *FAAH* genes. Identical nucleotides are marked with an *asterisk*. *Left-* and *right-handed arrows* denote SP1 sites in the − and + strands respectively. *Oval box*, CRE-like site; *rectangular box*, estrogen-responsive element (*ERE*) site. *B*, gel shift and supershift (*wt + Ab*) experiments were performed with 3 μg of T lymphocytes nuclear extracts prepared before (−) and after (+) stimulation with 10 nM leptin. Shifted and supershifted complexes are indicated with a *big* and a *small arrow* respectively. Oligonucleotides as ^{32}P-labeled probes contained mutated (*mutL*) or wild-type (*wt*) CRE-like site.

DISCUSSION

In this study we show that leptin stimulates FAAH activity and expression in human T lymphocytes through a leptin receptor-mediated activation of STAT3 signaling, which leads to up-regulation of a CRE-like site in FAAH promoter. Moreover, by using leptin-deficient (*ob/ob*) mice we also show that leptin tonically controls FAAH activity *in vivo*, which opens new avenues for the management of immune and fertility defects under leptin control in humans.

Leptin modulates FAAH activity and expression in T-cells at the same circulating levels shown to unbalance cytokine production from these cells (4). These concentrations of L were found to saturate the binding sites in T lymphocytes (Fig. 3*A*) with calculated binding constants (K_d and B_{max}) similar to those of the leptin receptor (7, 30). This observation together with the ability of cold L to fully displace ^{125}I-labeled L strongly suggest that T-cells have an authentic LR, thus extending a previous study that demonstrated that the same cells express the mRNA for the long isoform of LR (4). These findings taken together with the observation that the effects of L on FAAH activity and expression were fully neutralized by anti-L antibodies and by soluble LR (Fig. 1*A*) strongly suggest that FAAH activation by L occurred through activation of LR. On the other hand, L was ineffective on CBR binding and AMT activity in T lymphocytes (Fig. 1*C*), suggesting that FAAH was the only "check point" for the effect of L. These observations extend previous studies on the role of FAAH, but not CB receptors or AEA transporter, in modulating immunoendocrine interactions in early pregnancy in humans (21, 22). They are also in keeping

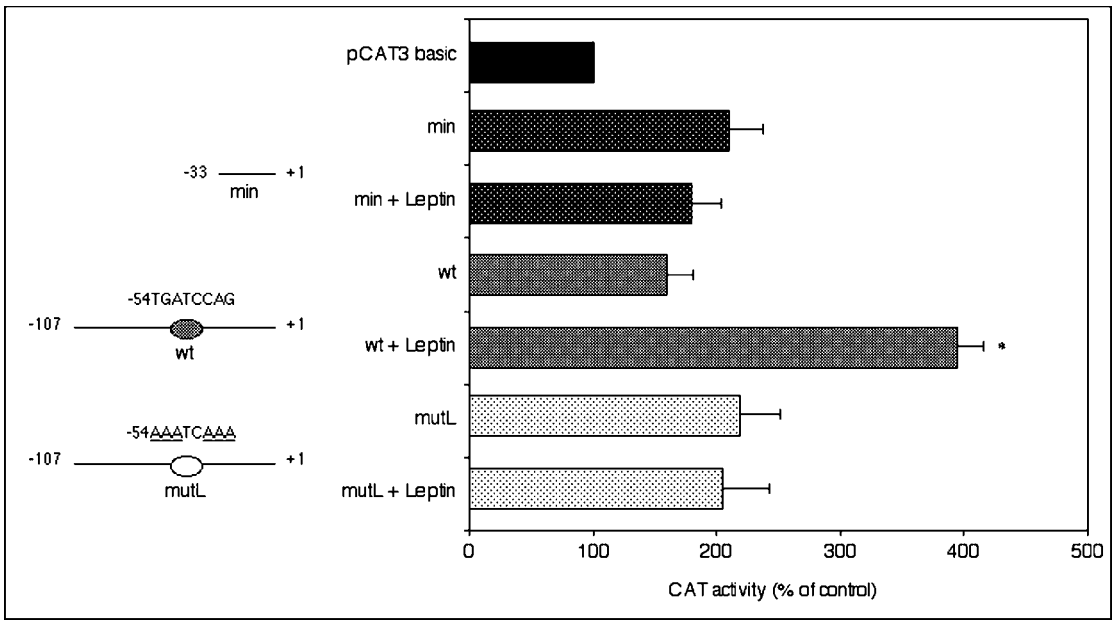
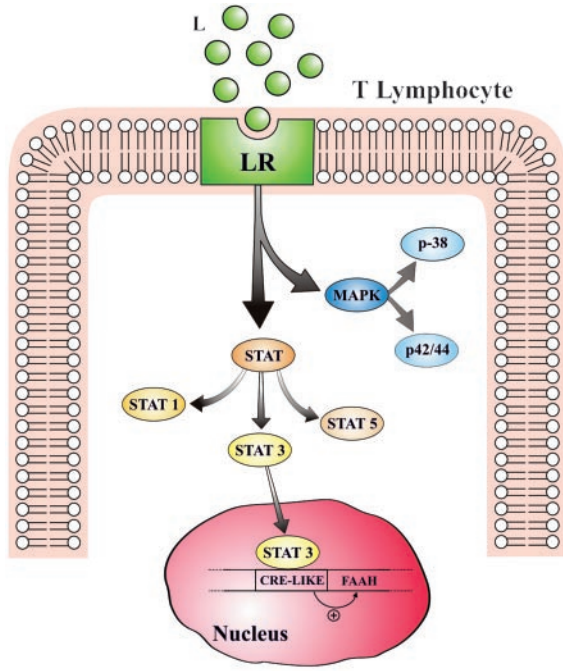


FIG. 6. **Analysis of FAAH promoter by transient expression.** The 5′ flanking regions of the human *FAAH* gene were cloned in the *PstI*/*Xba*I sites of pCAT3-basic vector. *Min* (*black bars*), −33 to +1 upstream region containing only the proximal SP1 site; *wt* (*dark gray bars*), −107 to +1 upstream region containing the two SP1 sites flanking a putative CRE-like site (*gray*); *mutL* (*light gray bars*), same as *wt* but with the mutated CRE-like sequence; mutated sites and nucleotides are in *white* and *underlined* respectively. T lymphocytes were transfected with the aforementioned constructs and left untreated or treated with 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 % with respect to the activity of the empty vector, pCAT3-basic, which was set to 100%. * denotes $p < 0.01$ *versus* control, and *vertical bars* represent S.D. values.

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 (23). In the same line, we found that 10 nM L, which increased FAAH in T lymphocytes up to ~300% over controls (Fig. 1*A*), reduced AEA levels in these cells down to ~15%.[2] 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-arachidonoylglycerol and oleoylethanolamide (12, 16). In addition, the reduction of FAAH activity and content in leptin-deficient (*ob*/*ob*) compared with wild-type mice and the ability of exogenous leptin to reverse this reduction (Table I) speak in favor of a tonic control of FAAH by L *in vivo*. This hypothesis is strengthened by the lack of effect of exogenous L in wild-type mice (Table I), which already have enough circulating L to saturate LR binding sites (1, 2). In this context, it seems noteworthy that AEA did not affect L binding to LR (Fig. 3*A*), suggesting that AEA could not directly contribute to the L-mediated regulation of its degradation by FAAH. At any rate, the up-regulation of FAAH expression by L is a major finding of this investigation and is associated with higher FAAH activity in T-cells.

The mechanism of FAAH activation by L was further investigated. Binding of L to the long isoform of LR is known to trigger different signaling pathways, which include STAT-dependent and/or MAPK-dependent signal transduction (7–11). In human T lymphocytes, physiological doses of L activated only STAT3 (Fig. 4, *A–E*), which is preferentially activated also in other cell types (7, 8, 31) and tissues (Ref. 1 and references therein). To clarify how STAT3 could up-regulate FAAH activity and expression, we analyzed 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



SCHEME 1. **Model of the activation of FAAH promoter by leptin in human T-cells.** L binds to its receptor LR in human T lymphocytes leading to the activation of STAT3 but not of other typical LR-dependent downstream signals like STAT1, STAT5, or MAPK, p38, p42, or p44. Phosphorylation of STAT3 results in the activation of a CRE-like region in the FAAH promoter, thus up-regulating gene expression.

(Fig 5*A*). The FAAH promoter did not contain STAT3 DNA binding motifs yet it did contain a CRE-like element between the two SP1 sites (Fig. 5*A*). Such CRE-like elements have been recently shown to be tethered by STAT3, thus leading to a novel type of up-regulation of gene transcription (31). Transfection experiments using FAAH promoter constructs with mu-

tated CRE-like elements (mutL) revealed that indeed these sites confer STAT3 responsiveness (Fig. 6). EMSA analysis and gel supershift assays further corroborated this conclusion (Fig. 5*B*). Therefore, it can be concluded that L, by binding to LR in human T lymphocytes, activates STAT3, which in turn up-regulates *FAAH* gene transcription by tethering to a CRE-like site in the FAAH promoter. The overall regulation of FAAH promoter by L in human T-cells is depicted in Scheme I.

It seems noteworthy that this is the first characterization of the promoter and the first description of the transcriptional regulation of human FAAH. As yet, two interesting reports have characterized the promoter (38) and the transcriptional regulation (35) 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). Remarkably, here we show that the human FAAH promoter does not contain an estrogen response element at the same position (Fig. 5*A*). In this context, we have recently shown that estrogen down-regulates FAAH in human cells according to a non-genomic mechanism (40). These observations suggest a relevant species specificity of FAAH regulation, though the human and mouse FAAH (localized on chromosome 1 and 4, respectively) share 84% sequence identity (25) and have a conserved genomic structure (34). 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 leptin and the peripheral endocannabinoid system and suggest that FAAH, but not anandamide transporter or CB receptors, might be the target for new therapies of human defects in immunity and fertility.

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Leptin Activates the Anandamide Hydrolase Promoter in Human T Lymphocytes through STAT3

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