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Regulation, Function, and Dysregulation of Endocannabinoids in Models of Adipose and β-Pancreatic Cells and in Obesity and Hyperglycemia

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Context: Cannabinoid CB_1 receptor blockade decreases weight and hyperinsulinemia in obese animals and humans in a way greatly independent from food intake.

Objective: The objective of this study was to investigate the regulation and function of the endocannabinoid system in adipocytes and pancreatic β-cells.

Design, Setting, and Patients: Mouse 3T3-F442A adipocytes and rat insulinoma RIN-m5F β-cells, pancreas and fat from mice with diet-induced obesity, visceral and sc fat from patients with body mass index equal to or greater than 30 kg/m^2, and serum from normoglycemic and type 2 diabetes patients were studied.

Main Outcome Measure: Endocannabinoid enzyme and adipocyte protein expression, and endocannabinoid and insulin levels were measured.

Results: Endocannabinoids are present in adipocytes with levels peaking before differentiation, and in RIN-m5F β-cells, where they are under the negative control of insulin. Chronic treatment of adipocytes with insulin is accompanied by permanently elevated endocannabinoid signaling, whereas culturing of RIN-m5F β-cells in high glucose transforms insulin down-regulation of endocannabinoid levels into up-regulation. Epididymal fat and pancreas from mice with diet-induced obesity contain higher endocannabinoid levels than lean mice. Patients with obesity or hyperglycemia caused by type 2 diabetes exhibit higher concentrations of endocannabinoids in visceral fat or serum, respectively, than the corresponding controls. CB_1 receptor stimulation increases lipid droplets and decreases adiponectin expression in adipocytes, and it increases intracellular calcium and insulin release in RIN-m5F β-cells kept in high glucose.

Conclusions: Peripheral endocannabinoid overactivity might explain why CB_1 blockers cause weight-loss independent reduction of lipogenesis, of hypoadiponectinemia, and of hyperinsulinemia in obese animals and humans. ***(J Clin Endocrinol Metab* 91: 3171–3180, 2006)**

THE ENDOCANNABINOID SYSTEM comprises two cannabinoid receptor subtypes, CB_1 and CB_2, their endogenous ligands [the endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG)], and enzymes for ligand biosynthesis and degradation, *i.e.* the *sn*-1-selective diacylglycerol-α lipase (DAGL-α), the monoacylglycerol lipase (MAGL), the *N*-arachidonoylphosphatidylethanolamine phospholipase D (NAPE-PLD), and the fatty acid amide hydrolase (FAAH) (1). Although discovered relatively recently, this signaling system has already provided a drug for the treatment of obesity: rimonabant, a selective CB_1 antagonist (2, 3). In fact, it is known that blockade of CB_1 receptors causes reduction of food intake in food-deprived lean animals, and in *ad lib*-fed obese animals (2, 4–6). Like other orexigenic mediators, hypothalamic endocannabinoids are subject to negative regulation by leptin and are permanently elevated in genetically obese rodents, in which they contribute to hyperphagia (7–9). However, it is also clear that the persistent weight loss that follows chronic blockade of CB_1 receptors is largely independent from its transient inhibition of food intake (5, 10, 11). CB_1-deficient mice exhibit significantly less fat mass that wild-type mice and are not susceptible to developing obesity after a high-fat diet, even when they consume as much food as wild types (12, 13). These findings suggest that the endocannabinoid system contributes to obesity via peripheral mechanisms, possibly by being permanently up-regulated, as recently shown in the liver of mice with diet-induced obesity (DIO) (14). Chronic blockade of CB_1 receptors in obese animals and humans also reduces, when present, the signs of the "metabolic syndrome" as first

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Abbreviations: 2-AG, 2-Arachidonoylglycerol; BMI, body mass index; DAGL-α, *sn*-1-selective diacylglycerol-α lipase; DIO, diet-induced obesity; FAAH, fatty acid amide hydrolase; IBMX, 3-isobutyl-1-methylxanthine; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-arachidonoylphosphatidylethanolamine phospholipase D; PPAR-γ, peroxisome proliferator-activated receptor γ.
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defined by the National Cholesterol Education Program Adult Treatment Panel III (15), *i.e.* high waist circumference (abdominal obesity), high triglyceridemia, low high-density lipoprotein cholesterol, high mean arterial pressure, and high fasting glycemia (and subsequent hyperinsulinemia) (10, 11, 16). Yet, it is not known whether these additional beneficial effects are merely the consequence of weight loss or also the result of peripheral actions of CB_1 blockers exerted directly on cells involved in lipogenesis and insulin production, *i.e.* white adipocytes and pancreatic islet β-cells, respectively. White adipocytes do express functional CB_1 receptors, whose levels are higher in obese rats and whose blockade leads to increased levels of adiponectin (13, 17), an adipokine that enhances insulin sensitivity and glucose and lipid metabolism (18). Pancreatic islets also express cannabinoid receptors (19). However, it is not known whether endocannabinoids are present in adipocytes or pancreatic β-cells, nor whether they directly contribute to lipogenesis and hyperinsulinemia during obesity and hyperglycemia. Thus, we investigated the function and possible dysregulation of endocannabinoids in isolated adipocytes and β-cells cultured under conditions mimicking either normal or unbalanced energy homeostasis, and in adipose tissue, pancreas, and serum of DIO mice and obese or type 2 diabetic hyperglycemic patients.

Subjects and Methods

Cell cultures

The mouse preadipocyte 3T3-F442A cell line (fifth to 10th passage) was kindly provided by Dr. Mohamed Bensaid from Sanofi-Aventis (Montpellier, France). 3T3-F442A preadipocytes were grown according the manufacturer's recommendations. After confluence, 3T3-F442A adipose differentiation was obtained in the presence of the same culture medium supplemented with 0.9 μM insulin. Cells were placed into culture (d −6) and grown to confluence (d −2) before stimulation with insulin (d 0). Terminal differentiation occurred by d 12. Differentiation was also induced with a mixture of 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), dexamethasone (1 mM), and insulin (0.9 μM). For adipocyte stimulation, HU210 (100 nM), rimonabant (SR141716A or SR1, 100 nM), SR144528 (SR2, a selective cannabinoid CB2 receptor antagonist, 100 nM), leptin (20 nM), and ciglitazone [a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist, 20 μM] were added in dimethyl sulfoxide (final concentration 0.1%) to the culture medium. For experiments on cAMP, confluent 3T3-F442A mature adipocytes were plated in six-well dishes and stimulated for 10 min at 37 C with 1 μM forskolin in 400 μl serum-free recommended medium containing 20 mM HEPES, 0.1 mg/ml BSA, and 0.1 mM IBMX. Cells were treated with vehicle, or HU-210 (20 or 200 nM), or with SR1 (200 nM) or with HU-210 + SR1 (200 nM each). After incubation, cAMP cellular content was determined by means of a cAMP assay kit (Amersham, Buckinghamshire, UK).

RIN-m5F rat insulinoma β-pancreatic cells were obtained from ATCC and grown according to the manufacturer's recommendations. For experiments with cells in low and high glucose conditions, cells were grown in the recommended culture medium containing respectively 2.4 g/liter glucose (13 mM) and 4.5 g/liter glucose (25 mM) for 24 h before experiments. For RIN-m5F cell stimulation, leptin (20 nM), insulin (100 nM), HU210 (200 nM), rimonabant (1 μM), and SR144528 (1 μM) were added in dimethyl sulfoxide (final concentration 0.1%) to the culture medium depleted in fetal calf serum. In these cells, the effect of HU-210 (50, 100, 1000, and 5000 nM) on intracellular Ca^{2+} in the presence or absence of SR1 (200 nM) was measured by means of Fluo-4 or Fura-2 fluorometric detection.

Insulin release quantification

Insulin release from RIN-m5F rat β-cells was quantified in the cell media by ELISA (Mercodia) realized by Biovendor (Modrice, Republic Tcheque).

Oil Red-O staining

Light microscopy and Oil Red-O staining were used to monitor the characteristic cell rounding and lipid droplet accumulation in these cells during differentiation (20).

Animals

Male lean and DIO C57BL/6J mice and ICR mice with streptozotocin-induced diabetes (21) were anesthetized and decapitated. DIO mice were obtained by feeding lean C57BL/6J mice with an increased fat diet (from 9 to 20% fat content) for 8 wk. Pancreas and epididymal fat mass were removed and immediately frozen in liquid nitrogen until quantitative determination of endocannabinoids. Blood was collected to determine leptin and insulin levels using RIA kits from Linco. Guidelines for the use and care of laboratory animals of the authors' institutions were followed.

Subjects and blood or fat sampling

Visceral/sc fat from normoweight and overweight/obese volunteers was collected during Roux-en-Y gastric Bypass surgery for all 20 obese patients, and during colecystectomy (n = 4) and hiatal hernias removal (n = 6) for the normoweight controls (Table 1). Samples were immediately frozen in liquid nitrogen until endocannabinoid quantification. Blood from patients with type 2 diabetes and age-matched healthy volunteers was collected between 0800 and 0900 h, the last treatment having been done not earlier than 12 h before blood sampling (Table 2). Regarding the determination of circulating preprandial and postprandial endocannabinoid levels, 12 healthy human subjects (eight males, four females) were recruited [age = 32.3 ± 3.9 yr; body mass index (BMI) = 21.7 ± 2.9 kg/m^2, means ± SD]. After a 12-h fasting period, volunteers received a high-fat meal providing 601.12 kcal and consisting of 16.6% protein, 39.25% carbohydrate, and 44.15% fat. Preprandial and postprandial blood samples were collected 1 h before and after the test meal, respectively. All patients and volunteers were informed of the study procedures and signed an informed consent.

Purification and quantification of endocannabinoids

The extraction, purification, and quantification of anandamide and 2-AG from tissues, blood, and cells was performed as described previ-

TABLE 1. Obese *vs.* normoweight patients

	Age (yr)	BMI (kg/m^2)	Gender	Glucose (mmol/liter)	Fat mass (kg)	Lean mass (kg)
Normoweight volunteers (n = 10) mean ± SE	56.7 ± 5.9	21.3 ± 0.3	4 male/6 female	4.83 ± 0.17	14.9 ± 2.7	43.6 ± 4.8
Obese patients (n = 20) mean ± SE	45.9 ± 2.1[a]	45.1 ± 1.8[b]	9 male/11 female	5.67 ± 0.11[c]	59.5 ± 17.2[b]	47.1 ± 12.1

None of the patients was under pharmacological treatment at the time of surgery. Fat and lean mass was assessed by bioimpedometric analyses.

[a] $P < 0.05$ *vs.* normoweight volunteers, as assessed by the Kuskal-Wallis nonparametric test.

[b] $P < 0.0005$ *vs.* normoweight volunteers, as assessed by the Kuskal-Wallis nonparametric test.

[c] $P < 0.005$ *vs.* normoweight volunteers, as assessed by the Kuskal-Wallis nonparametric test.

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TABLE 2. Type 2 diabetic *vs.* healthy volunteers

	Age (yr)	BMI (kg/m^2)	Gender	Glucose (mmol/liter)	Cholesterol (mmol/liter)	Triglycerides (mmol/liter)
Healthy volunteers (n = 8) mean ± SE	62.3 ± 2.4	28.6 ± 1.9	5 male/3 female	5.72 ± 0.22	4.56 ± 0.56	0.93 ± 0.10
Diabetic patients (n = 10) mean ± SE	69.0 ± 4.0	33.5 ± 3.0	4 male/6 female	10.28 ± 1.17[a]	5.95 ± 0.97[b]	2.00 ± 0.25[a]

This experiment was designed to assess whether or not a noncorrected hyperglycemia, due to a pathological condition, results in increased serum endocannabinoid levels. For this reason we used patients with type 2 diabetes with randomized pharmacological treatments. In fact, of the 10 patients, two were under treatment with metformin, one with metformin + insulin, one with glibenclamide, two with metformin + glibenclamide, one with acarbose + glibenclamide, and three with insulin only. In each case, the last treatment was given not later than 12 h prior to blood sampling. As shown in the table, this heterogeneity in treatment resulted in similar fasting glycemia values.

[a] $P < 0.005$, *vs.* healthy volunteers, as assessed by the Kuskal-Wallis nonparametric test.

[b] $P < 0.001$, *vs.* healthy volunteers, as assessed by the Kuskal-Wallis nonparametric test.

ously (22). After extraction, endocannabinoid fractions were subjected to isotope-dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometric analysis as previously described (22).

Statistical analysis

All results were expressed as mean ± SEM. The statistical significance of differences in mean values was assessed by one-way ANOVA followed by the Bonferroni's *post hoc* analysis, or by the Kruskal-Wallis nonparametric test in the case of human samples.

Real-time RT-PCR analyses

Real-time cDNA quantification was performed by a thermocycler iCycler iQ (Bio-Rad, Hercules, CA). Fluorescence data were collected during elongation. Optimized PPAR-γ, adiponectin, CB_1, CB_2, FAAH, NAPE-PLD, DAGL-α, and MAGL primers for SYBR Green analysis (and relative TaOpt), were designed by "Beacon Designer" software and synthesized by MWG-Biotech AG (Ebersberg, Germany). Assays were performed in triplicate (SD of threshold cycle mean < 0.5) and a standard curve from consecutive 5-fold dilutions (150–0.24 ng) of a cDNA pool representative of all samples, was included for each determination. Relative expression analysis correct for PCR efficiency and normalized respect to reference genes β-actin and hypoxanthine phosphoribosyltransferase was performed by "REST C" software for group-wise comparison and statistical analysis.

Results

Regulation of the endocannabinoid system during adipocyte differentiation

Mouse 3T3 F442A preadipocytes were differentiated into adipocytes using conditions mimicking hyperinsulinemia, with a high concentration of insulin, either alone or in a mixture with IBMX and dexamethasone. Differentiation was monitored by measuring PPAR-γ expression by real-time PCR (fold enhancement over d 0) and Oil Red-O staining of lipid droplets, and occurred more rapidly with insulin alone (Fig. 1A). PPAR-γ expression was significantly different from d 0 on d 8 and 12 under both conditions. Note the full cell rounding occurring already at d 8 with insulin only, and the increased formation of lipid droplets (red dots, *black* in Fig. 1A) between d 8 and 12. Basal and forskolin-induced cAMP levels were also increased 3.5 ± 0.2- and 3.0 ± 0.3-fold at d 8 *vs.* d 0. Adiponectin expression was increased 5.9 ± 0.6-fold at d 12 *vs.* d 0 (data not shown, means ± SE). Irrespective of the type of stimulation, adipocyte differentiation was preceded by a 2.5- to 7-fold enhancement of the levels of the two endocannabinoids, anandamide and 2-AG, the latter of which remained strongly elevated in mature (d 8) and hypertrophic (d 12) adipocytes (Fig. 1B). In fact, although peaks of 2-AG levels were seen, depending on the stimulus, at d 4 or 8, there was no statistically significant difference in 2-AG levels between d 4, 8, and 12. Accordingly, differentiation was accompanied by a dramatic increase of the expression of the 2-AG biosynthesizing enzyme, the DAGL-α (1), whereas the 2-AG-degrading enzyme, MAGL, was unchanged (Fig. 1C). The expression level of the anandamide biosynthesizing enzyme, NAPE-PLD, decreased during differentiation and was 1.25 ± 0.01-, 1.37 ± 0.01-, and 0.85 ± 0.01-fold *vs.* d 0 at d 4, 8, and 12, respectively, whereas that of the anandamide-degrading enzyme, FAAH, instead increased by going from 0.96 ± 0.04- to 1.31 ± 0.04- and 1.36 ± 0.03-fold *vs.* d 0 at d 4, 8, and 12, respectively (means ± SE, n = 4) (data not shown). These changes might explain why anandamide levels peaked at d 4 and decreased thereafter. CB_1 and CB_2 receptors were strongly expressed only in differentiating adipocytes and remained expressed in mature cells (Fig. 1C). CB_1 levels slightly decreased in hypertrophic *vs.* partially differentiated preadipocytes (d 8–12 *vs.* d 4).

Regulation of endocannabinoid levels by leptin and PPAR-γ

As previously reported in the rodent hypothalamus, lymphocytes, and uterus (7, 23, 24), both anandamide and 2-AG levels in mature adipocytes (d 8, with insulin only) were decreased after either acute (1 h) or prolonged (24 h) stimulation with leptin (20 nM) (Fig. 1D). In partially differentiated preadipocytes (d 4, with insulin only), leptin (20 nM, 1 h) decreased anandamide levels (from 5.7 ± 0.1 to 3.6 ± 0.4 pmol/mg lipid extract, n = 4, $P < 0.01$), but not 2-AG levels (from 41.0 ± 4.2 to 44.1 ± 5.5 pmol/mg lipid extract, n = 4). Ciglitazone (20 μM, 24 h), a selective agonist of PPAR-γ, decreased 2-AG, but not anandamide, levels in partially differentiated preadipocytes (d 4) (from 37.0 ± 7.0 to 13.4 ± 1.4 pmol/mg lipid extract, n = 4, $P < 0.05$), but not in mature adipocytes (d 8) (from 21.0 ± 2.2 to 22.2 ± 2.5 pmol/mg lipid extract, n = 4).

Effect of CB_1 stimulation on PPAR-γ and adiponectin expression and lipid levels in adipocytes

Chronic activation of cannabinoid receptors with HU-210 stimulated an early marker of adipocyte differentiation, PPAR-γ, at d 8, and inhibited adiponectin expression, a late marker of differentiation, only at d 12 (Fig. 2B). These effects were attenuated or reversed by rimonabant. HU-210 (200 nM) stimulated the accumulation of lipid droplets at d 8. The number of Oil Red-O-stained droplets per square millimeter (in *black* in Fig. 2A) were 7,590 ± 310, 11,670 ± 470, 6,830 ± 90, and 9,170 ± 540 with vehicle, HU-210 ($P < 0.01$ *vs.*

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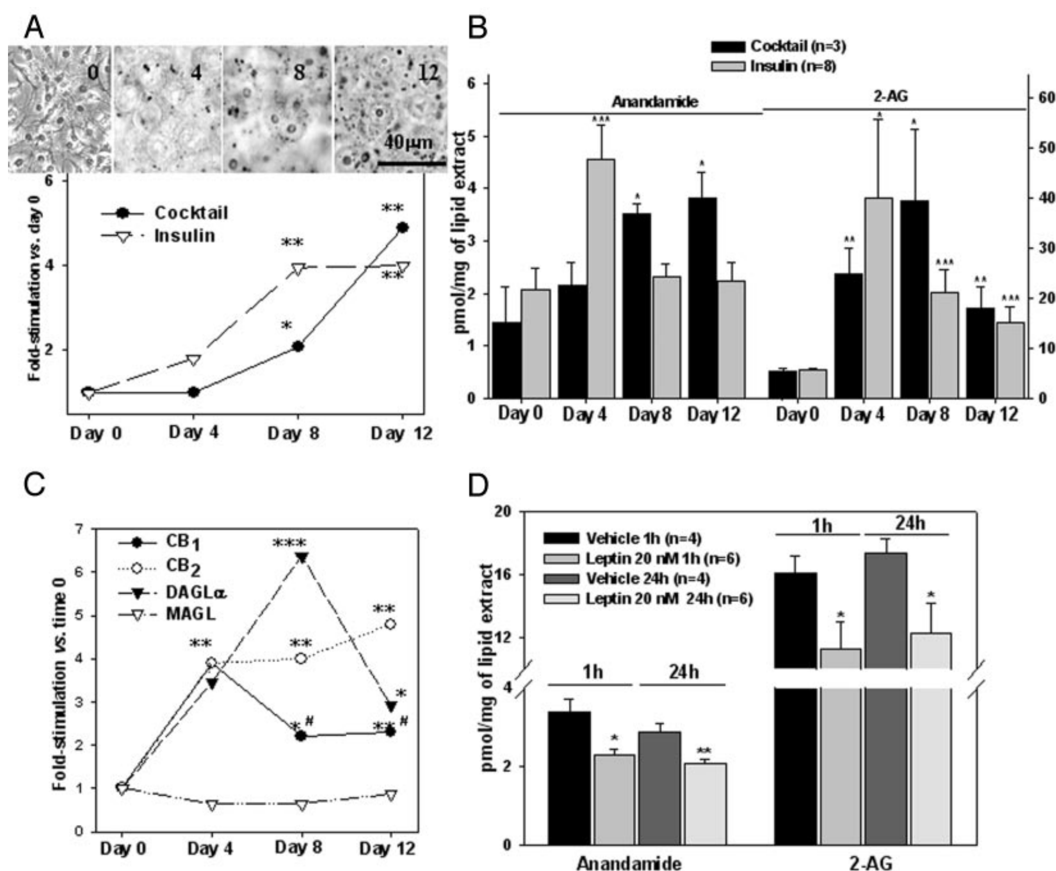

FIG. 1. Regulation and role of the endocannabinoid system during adipocyte differentiation. A, Differentiation of mouse 3T3 F442A preadipocytes into adipocytes was monitored by measuring PPAR-γ expression by real-time RT-PCR (*lower panel*), or Oil Red-O staining under a microscope (*upper panel*, insulin only). mRNA expression is expressed as fold enhancement over d 0. Error bars are not shown, and they were always equal to or less than 10%. SD values for cycle threshold were always less than 1%. *, **, $P < 0.05$, 0.01 *vs.* d 0, respectively, as assessed by ANOVA followed by the Bonferroni's test. B, Levels of endocannabinoids during adipocyte differentiation induced with insulin alone (0.9 μM) or in a mixture with IBMX (0.5 mM) and dexamethasone (1.0 mM). Endocannabinoid levels were measured by isotope-dilution liquid chromatography-mass spectrometry (see *Subjects and Methods*). Data are means ± SE of n = 3–6 separate experiments. *, **, ***, $P < 0.05$, 0.01, 0.005 *vs.* vehicle, respectively, as assessed by ANOVA followed by the Bonferroni's test. C, Effect of chronic stimulation with insulin alone (0.9 μM) on the expression of CB_1, CB_2, DAGL-α, and MAGL, as measured by real-time RT-PCR (fold enhancement over d 0). Error bars are not shown because they were always equal to or less than 10%. SD values for cycle threshold were always less than 1%. *, **, ***, $P < 0.05$, 0.01, 0.005 *vs.* d 0, respectively; #, $P < 0.05$ *vs.* d 4, as assessed by ANOVA followed by the Bonferroni's test. D, Effect on endocannabinoid levels of acute (1 h) or prolonged (24 h) stimulation with leptin (20 nM) of differentiated adipocytes (d 8, insulin only). Data are means ± SE of n = 4–6 separate experiments. *, **, $P < 0.05$, 0.01 *vs.* vehicle, respectively, as assessed by ANOVA followed by the Bonferroni's test.

vehicle), HU-210 plus rimonabant ($P < 0.01$ *vs.* HU-210) and rimonabant alone ($P > 0.05$ *vs.* vehicle), respectively (means ± SE, n = 4). HU-210 also inhibited forskolin-induced cAMP formation (EC_{50} = 145 ± 18 nM, 73.0 ± 1.5% inhibition at 200 nM, $P < 0.005$), in a way reversed by rimonabant (200 nM, 82.0 ± 6.0% inhibition of HU-210 effect, $P < 0.005$, means ± SE, n = 3) (data not shown). Stimulation of lipid droplets with HU-210 was also observed at d 4. In this case, the number of red-stained droplets per square millimeter was 4570 ± 350, 8470 ± 340, 7130 ± 190, and 8280 ± 250 with vehicle, HU-210 ($P < 0.01$ *vs.* vehicle), HU-210 together with rimonabant ($P < 0.05$ *vs.* HU-210) and HU-210 together with SR144528 ($P > 0.05$ *vs.* HU-210), respectively.

Regulation of the endocannabinoid system in rat RIN-m5F β-pancreatic cells

Rat insulinoma RIN-m5F β-cells are a widely used model of pancreatic islet β-cells in as much as they release insulin and also respond to this hormone (25). We found that they express both CB_1/CB_2 receptors and enzymes for endocannabinoid biosynthesis and metabolism (Fig. 3, A and B). With respect to 3T3 F442A adipocytes (d 4), RIN cells expressed 2.7 ± 0.03-fold more and 5.4 ± 0.34-fold less CB_1 and CB_2 receptor mRNA, respectively (means ± SE, n = 5), although this comparison can only be made on the assumption that different primers (for different animal species) work with similar efficacy. The expression of CB_1 and CB_2 in RIN cells was confirmed by Western immunoblots, which showed bands at approximately 83, 53, and 43 kDa for CB_1, and approximately 45 and 39 kDa for CB_2 (data not shown), in agreement with the expected sizes of the native and post-translationally modified receptors. Enzymes catalyzing anandamide biosynthesis and hydrolysis, NAPE-PLD and FAAH, and 2-AG biosynthesis and hydrolysis, DAGL-α and MAGL, were identified. Near-confluent cells at a low number of passages were kept either in "high" (25 mM) or "low"

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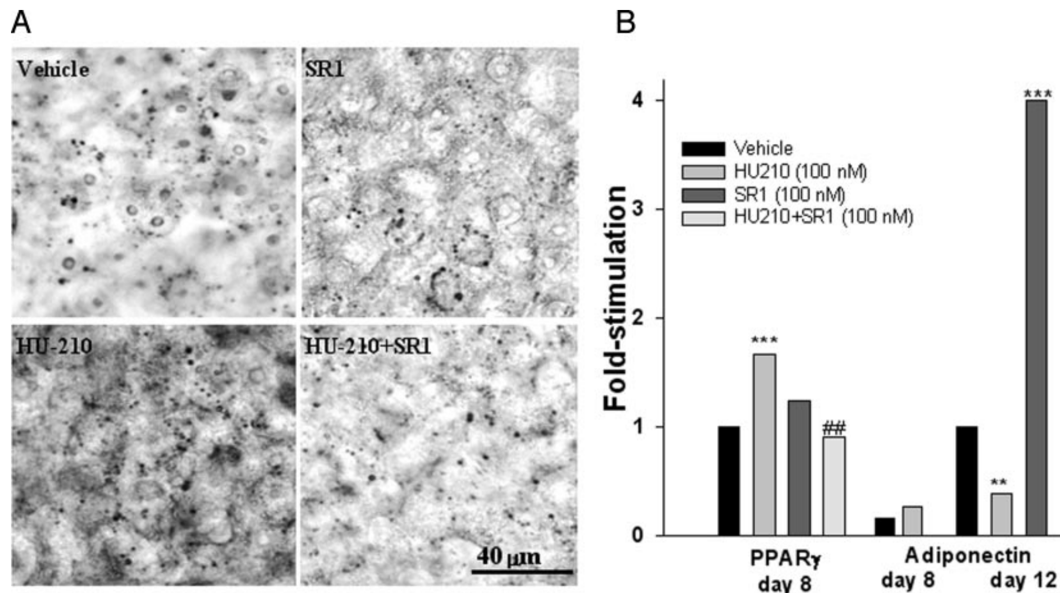

FIG. 2. Effect of CB_1 stimulation on PPAR-γ and adiponectin expression and on lipogenesis in adipocytes. A, Effect of chronic treatment with the potent CB_1/CB_2 agonist, HU-210 (100 nM), with or without coincubation with rimonabant (SR1, 100 nM) on lipid droplet formation in differentiated adipocytes, as revealed by Oil Red-O staining and microscope observation (d 8, insulin only). Drugs were added to each change of medium, every other day, until d 8 (see *Results*). B, Effect on PPAR-γ and adiponectin expression, in differentiated and mature adipocytes (obtained with insulin only), respectively, of chronic treatment with HU-210 (100 nM), rimonabant (SR1, 100 nM), and HU-210 + rimonabant (only with PPAR-γ, because, in the case of adiponectin, rimonabant was active *per se* also at lower concentrations). Drugs were added to each change of medium, every other day, until d 8 for PPAR-γ and until d 12 for adiponectin. Expression was measured by real-time RT-PCR and is expressed as fold enhancement over vehicle. In the case of adiponectin, the results obtained after incubation with HU-210 (100 nM) until d 8 are also shown and are expressed as fold expression *vs.* the vehicle at d 12. Error bars are not shown and they were always less than or equal to 10%. SD values for cycle threshold were always less than 1%. **, ***, $P < 0.01$, 0.005 *vs.* vehicle, respectively; ##, $P < 0.01$ *vs.* HU-210 (P values were assessed as described in *Subjects and Methods*, real-time RT-PCR).

(13 mM) glucose for 24 h before stimulation with a 2-h "pulse" of 33 mM glucose, which, under both conditions, elevated both anandamide and 2-AG levels (Fig. 3, C and D). In β-cells kept on low glucose, which exhibited significantly lower endocannabinoid levels, costimulation with insulin abolished 33 mM glucose-induced endocannabinoid elevation (Fig. 3C). Conversely, in β-cells kept on high glucose to mimic hyperglycemic conditions, insulin not only did not inhibit 33 mM glucose-induced endocannabinoid elevation, but also enhanced endocannabinoid levels *per se* (Fig. 3D). Finally, under both conditions, leptin only decreased 2-AG, but not anandamide, levels and only after prolonged (24 h) stimulation (Fig. 3, C and D, and data not shown).

Effect of CB_1 stimulation on insulin release from RIN-m5F β-pancreatic cells

In β-cells kept on high glucose, but not in those kept in low glucose (data not shown; see also Ref. 19 for data in mouse β-cells), stimulation of cannabinoid receptors with HU-210 enhanced insulin release, as assessed by a specific ELISA. This effect was reversed by rimonabant but not by a cannabinoid CB_2 receptor antagonist (Fig. 3E and data not shown), and was likely due to elevation of intracellular Ca^{2+} by HU-210, which occurred within a similar range of concentrations ($EC_{50} = 260 \pm 31$ nM, n = 3) and was antagonized by rimonabant (Fig. 3F).

Dysregulation of endocannabinoid levels in obese mice and humans

DIO mice exhibited significantly higher ($P < 0.0005$) body weight and insulin or leptin levels than lean mice (33.5 ± 1.0 *vs.* 23.2 ± 0.3 g; 1.11 ± 0.05 *vs.* 0.59 ± 0.02 ng/ml; 15.6 ± 1.26 *vs.* 5.94 ± 0.16 ng/ml, respectively). In these mice, pancreatic anandamide and 2-AG levels were higher than in lean mice (Fig. 4A). In contrast, in a mouse model of type 1 diabetes, no significant variation of pancreatic endocannabinoid levels was observed (Fig. 4C). Furthermore, in the visceral fat of both DIO mice and obese patients, we observed a significant increase of 2-AG, but not anandamide, levels (Fig. 4, B and D). In DIO mice, a 2.5-fold enhancement of 2-AG was observed in the epididymal fat. In obese patients (Table 1), an almost 2-fold increase was observed in the visceral fat (Fig. 4D). This difference was accounted for by male (52.2 ± 0.9 pmol/g, n = 4, *vs.* 98.1 ± 1.2 pmol/g, n = 9, $P < 0.05$) rather than female patients (71.3 ± 2.0 pmol/g, n = 6, *vs.* 100.8 ± 2.6 pmol/g, n = 11, $P > 0.05$), although no statistically significant difference existed between male or female patients. No significant differences between obese and normoweight were found by real-time PCR in the expression of the mRNAs of DAGL-α (−0.1 ± 0.05-fold in obese *vs.* normoweight, $P > 0.1$) or MAGL (1.1 ± 0.3-fold in obese *vs.* normoweight, $P > 0.1$), suggesting that the differences in 2-AG levels might be due to different availability of biosynthetic precursors, as shown previously for the up-regulation of hypothalamic 2-AG levels in obese rodents (7). Visceral fat of obese patients

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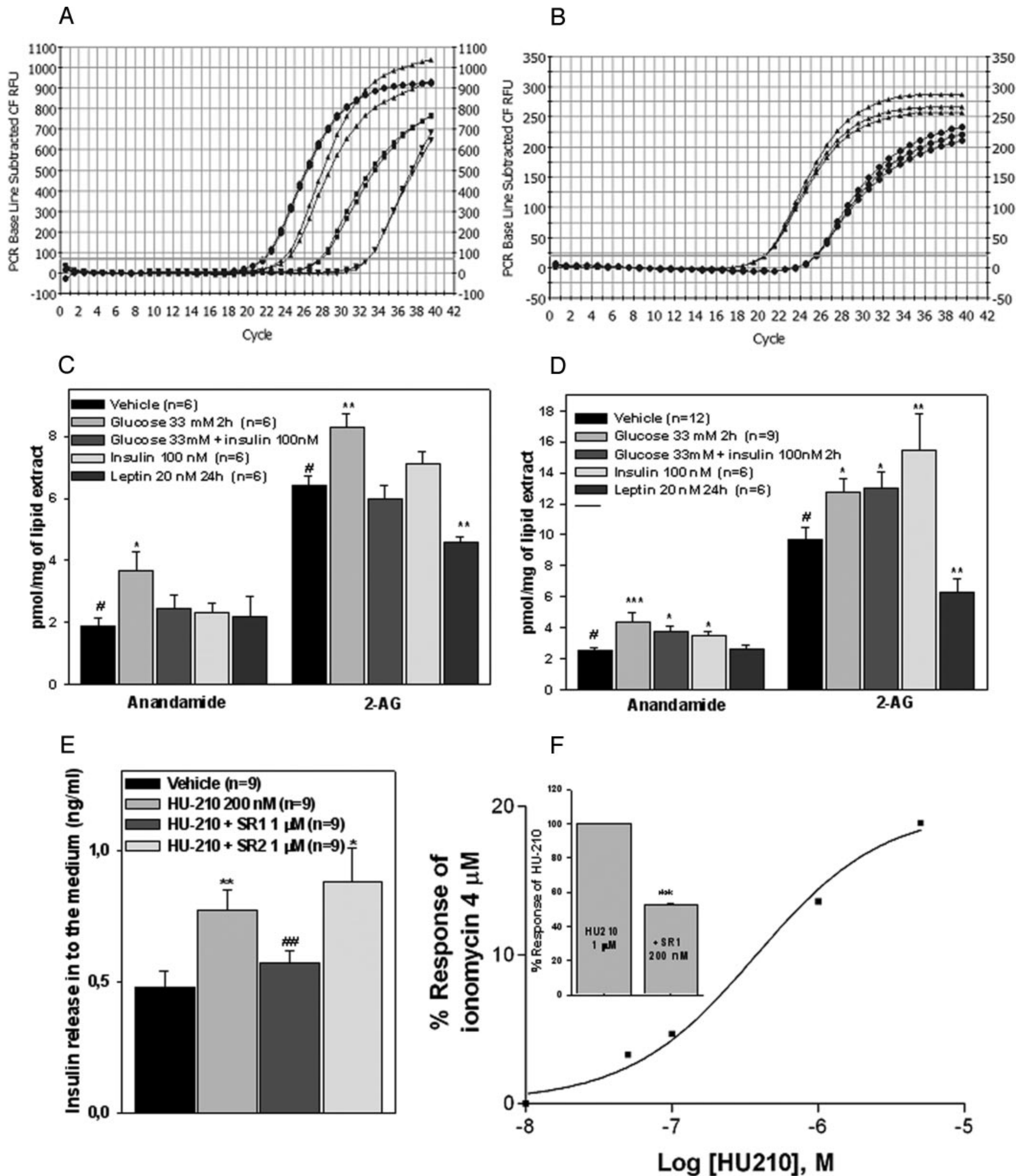

FIG. 3. Occurrence, regulation, and function of the endocannabinoid system in rat RIN-m5F β-pancreatic cells. A, Amplification profiles of CB_1 (*squares*), CB_2 (*downward triangles*), FAAH (*full circles*), and NAPE-PLD (*upward triangles*) target genes generated by real-time RT-PCR on RIN-m5F cell RNA. B, Amplification profiles of DAGL-α (*upward triangles*) and MAGL (*full circles*) target genes generated by real-time RT- PCR on RIN-m5F cell RNA. PCR efficiency was more than 92% and comparable for all the primers tested. C, Levels of endocannabinoids in RIN-m5F β-cells kept on low (13 mM) glucose for 24 h before stimulation with glucose (33 mM, 2 h), insulin (100 nM, 2 h), leptin (20 nM, 1 h), or glucose + insulin (2 h). Data are means ± SE of n = 6 separate experiments. *, **, $P < 0.05$, 0.01 *vs.* vehicle, respectively, as assessed by ANOVA followed by the Bonferroni's test. #, Significantly different from cells grown in 25 mM glucose. D, Levels of endocannabinoids in RIN-m5F β-cells kept on high (25 mM) glucose for 24 h

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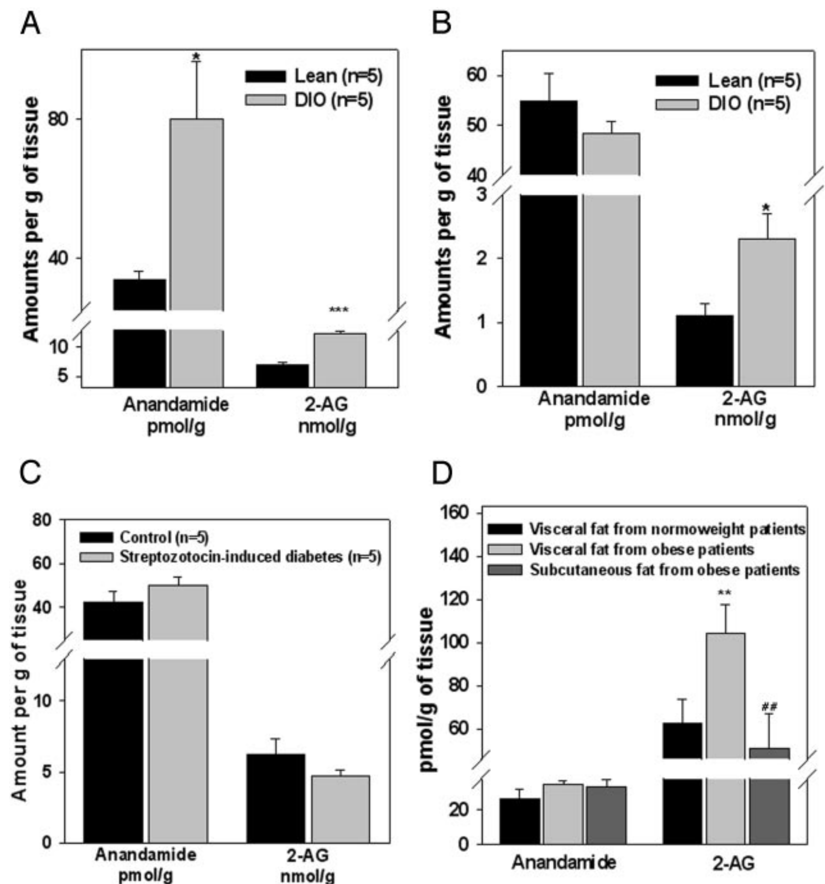

FIG. 4. Dysregulation of endocannabinoid levels in obese mice and humans. A, Endocannabinoid levels in the pancreas of lean and mice with DIO. *, ***, $P < 0.05, 0.005$ *vs.* controls, respectively, as assessed by ANOVA followed by the Bonferroni's test. B, Endocannabinoid levels in the epididymal fat of lean and DIO mice. *, $P < 0.05$ *vs.* controls, as assessed by ANOVA followed by the Bonferroni's test. C, Endocannabinoid levels in the pancreas of streptozotocin-treated mice, a model of type 1 diabetes. D, Endocannabinoid levels in the visceral adipose tissue of normoweight and overweight/obese humans and in the sc fat of obese patients (Table 1). **, $P \leq 0.01$ *vs.* visceral fat from normoweight volunteers; ##, $P < 0.01$ *vs.* visceral fat from obese patients as assessed by the Kuskal-Wallis nonparametric test.

also contained significantly higher 2-AG levels than sc fat ($P < 0.01$) (Fig. 4D). A trend toward decrease of CB_1 receptor levels (-1.8 ± 0.3-fold in obese *vs.* normoweight, $P = 0.08$) was found in the visceral fat obese patients.

Regulation and dysregulation of endocannabinoid levels in human blood during hyperglycemia

To assess whether a transient hyperglycemia, immediately corrected by a well-functioning insulin, results in decreased serum endocannabinoid levels, we measured the serum endocannabinoid levels in 12 normoweight adults 1 h before (preprandial) and after the meal (postprandial), respectively, when insulin levels increase from 4.02 ± 0.63 to 25.2 ± 6.47 mU/ml (means ± SE, $P < 0.005$). We observed a strong decrease of blood anandamide levels in postprandial volunteers (Fig. 5A). In a second experiment, we decided to evaluate whether a noncorrected hyperglycemia, due to a pathological condition, results in increased serum endocannabinoid levels. We used overweight male and female individuals with type 2 diabetes under randomized pharmacological treatments and whose only common clinical features were hyperglycemia of approximately 10 mmol/liter, age of approximately 69 yr, and BMI of approximately 33 kg/m^2 (Table 2). In this case, we detected higher circulating levels of both anandamide and 2-AG in diabetic patients than in healthy patients (Fig. 5B).

Discussion

The findings reported in this study indicate that a dysregulated endocannabinoid system in the adipocytes and

before stimulation with glucose (33 mM, 2 h), insulin (100 nM, 2 h), leptin (20 nM, 1 h), or glucose + insulin (2 h). Data are means ± SE of n = 6–9 separate experiments. *, **, ***, $P < 0.05, 0.01, 0.005$ *vs.* vehicle, respectively; #, $P < 0.05$ *vs.* vehicle of cells grown in high (13 mM) glucose, as assessed by ANOVA followed by the Bonferroni's test (see C). Note that low, high, and very high glucose do not refer to, nor do they reflect, the fasting concentrations of glucose occurring in humans during normo- or hyperglycemia, respectively. They refer instead to the optimal culturing conditions of RIN-m5F β-cells, which the manufacturer advises to grow in 25 mM glucose. E, Effect of 2 h of stimulation with HU-210 (200 nM), HU-210 + rimonabant (SR1, 1 μM), or HU-210 + SR144528 (SR2, 1 μM, a selective CB_2 antagonist) on insulin release from RIN-m5F β-cells kept on high (25 mM) glucose for 24 h. Insulin was measured using an established ELISA. *, **, $P < 0.05, 0.01$ *vs.* vehicle, respectively; ##, $P < 0.01$ *vs.* HU-210, as assessed by ANOVA followed by the Bonferroni's test. F, Effect of HU-210 on intracellular Ca^{2+} in RIN-m5F β-cells kept on high (25 mM) glucose for 24 h before stimulation. The effect of increasing concentrations of HU-210, measured with Fluo-4, 30 sec after administration, when the effect reaches a plateau before decreasing, is reported as the percentage of the maximal effect caused by ionomycin (4 μM), given to cells after HU-210. Similar potency was observed with HU-210 when the experiment was performed in the absence of extracellular Ca^{2+} and with Fura-2. F, *inset*, Effect of SR1 (200 nM) on HU-210 (1 μM)-induced effect on intracellular Ca^{2+}. The results are expressed as the percentage of the effect observed with HU-210 (1 μM) alone. Data are means ± SE of n = 3 separate experiments. **, $P < 0.01$, *vs.* HU-210 only, as assessed by ANOVA followed by the Bonferroni's test.

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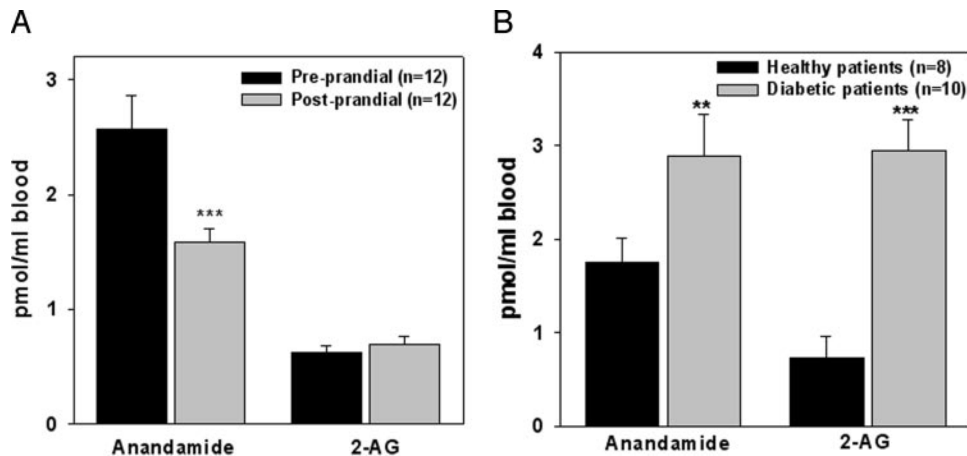

FIG. 5. Dysregulation of blood endocannabinoid levels in hyperglycemia. A, Serum endocannabinoid levels in postprandial *vs.* preprandial healthy normoweight volunteers. Blood sampling was carried out 1 h before and after the meal, respectively. B, Serum endocannabinoid levels in overweight type 2 diabetes *vs.* healthy volunteers. This experiment was designed uniquely to assess whether a noncorrected hyperglycemia, due to a pathological condition, results in increased serum endocannabinoid levels. For this reason, we purposely used male and female patients with type 2 diabetes under randomized pharmacological treatments and whose only common clinical features were hyperglycemia approximately 1.85 g/liter, age approximately 65, and BMI approximately 30 (Table 2). **, ***, $P < 0.01$, 0.005 *vs.* controls, respectively, as assessed by the Kuskal-Wallis nonparametric test (B) or the two-tailed paired Student's test (A).

β-cells might contribute to hyperlipidemia, hypoadiponectinemia, and hyperinsulinemia in obesity (26), and explain why CB_1 receptor blockade reduces these metabolic parameters in obese individuals independently not only from inhibition of food intake but also, in part, of body weight loss (27–29).

The data obtained in our model of adipocytes suggest that the endocannabinoid system is up-regulated immediately before adipocyte differentiation, possibly to concur at inducing differentiation and (also via CB_1-mediated inhibition of cAMP formation) lipogenesis. Endocannabinoids might then be "turned off" by leptin, which is produced by mature adipocytes and was shown here to efficaciously reduce both anandamide and 2-AG levels, and in part by PPAR-γ, which lowers 2-AG levels, although only in the early phase of adipocyte differentiation. However, in the continued presence of insulin, as during the hyperinsulinemia typical of obesity, adipocytes become hypertrophic and still contain high levels not only of CB_1 receptors, but also of 2-AG, because of the sustained expression of the major 2-AG biosynthesizing enzyme (and possibly also due to loss of inhibition by PPAR-γ). Only under these conditions, overstimulation of CB_1 receptors depresses the expression of a late marker of differentiation, adiponectin [as suggested here and previously (17, 30)], thereby contributing to the metabolic consequences of obesity (18) and explaining why blockade of CB_1 receptors restores in DIO mice a lean phenotype in terms of expression of adiponectin-dependent enzymes involved in lipid and glucose metabolism (31). There is a temporal separation between the effect of CB_1 stimulation on PPAR-γ and adiponectin (Fig. 2B). Therefore, although adiponectin expression is under the positive control of PPAR-γ, it is likely that the effect on adiponectin is independent from that on PPAR-γ. Accordingly, Gary-Bobo *et al.* (30) showed that a CB_1 antagonist does not induce lipid droplet formation in 3T3 adipocytes but still up-regulates late markers of differentiation.

In a model of pancreatic islet β-cells, we found that conditions mimicking hyperglycemia also lead to dysregulation of endocannabinoid signaling. A "very high" concentration of glucose always elevates the levels of both anandamide and 2-AG in these cells, whereas leptin is a much less efficacious inhibitor of endocannabinoid levels in these cells than in adipocytes. However, whereas in cells grown in low glucose, insulin reduces the glucose-induced elevation of endocannabinoid levels and has no effect *per se*, in β-cells kept in high glucose, endocannabinoid levels are not depressed any longer by insulin, which instead elevates both anandamide and 2-AG levels *per se*. Altogether, these data suggest that under conditions of hyperglycemia, such as during prediabetes, type 2 diabetes, and obesity, the endocannabinoid system in β-cells, instead of remaining under insulin control, becomes dysregulated. The consequent overstimulation of CB_1 receptors might reinforce insulin release—as shown here with HU-210—and cause permanent hyperinsulinemia. This, in turn, would: 1) start a vicious circle and further elevate endocannabinoid levels in β-cells, and 2) trigger adipocyte hypertrophy and endocannabinoid hyperactivity in adipocytes, with subsequent increase in lipid levels, decrease in adiponectin levels, and their metabolic consequences (supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

As previously observed in the brain (32, 33), peripheral anandamide and 2-AG levels are regulated in different ways by the same stimuli. This is not surprising in consideration of the fact that these compounds are biosynthesized and metabolized via different pathways (1, 2). For example, leptin controls the levels of anandamide through regulation of FAAH, and of 2-AG through regulation of the levels of its biosynthetic precursor (7, 23, 24). The differential regulation of endocannabinoid levels during adipocyte differentiation is also not surprising in view of findings in other differentiating cells or organs (33, 34).

The up-regulation of the levels of 2-AG in adipocytes, and

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of both endocannabinoids in β-cells, observed here under conditions of hyperinsulinemia/hyperglycemia, correspond to the scenario found *in vivo* in mice with DIO, where the levels of 2-AG were 2.5-fold higher in epididymal fat, and of both endocannabinoids were doubled in the pancreas, compared with lean mice. Conversely, no change in endocannabinoid levels was observed in the pancreas from streptozotocin-treated mice, an animal model of type 1 diabetes, indicating that, in this organ, endocannabinoid up-regulation is not simply due to islet β-cell malfunctioning. An almost 2-fold elevation of 2-AG, but not anandamide, levels was observed also in the visceral adipose tissue of overweight/obese patients with mild hyperglycemia compared with normoweight controls. In these patients, visceral fat contained significantly higher levels of 2-AG than sc fat, thus emphasizing the potential implication of the endocannabinoid system in the link between the metabolic syndrome and visceral, but not sc, adiposity (26). As 2-AG is significantly more efficacious than anandamide at CB_1 receptors (1), this up-regulation is likely to cause a significant elevation of adipocyte CB_1 receptor activity during obesity, thus possibly explaining the weight loss-independent effects of rimonabant on adiponectin levels (27).

The dysregulation of endocannabinoid levels in the adipose tissue and pancreas shown here, and previously in the liver (14), to be a hallmark of obesity may also concern other organs involved in the control of energy balance, as suggested by the recent finding of a strong correlation between human obesity and genetic malfunctioning of anandamide degradation (35). Therefore, higher endocannabinoid levels might be found also in the blood, as recently shown for obese *vs.* normoweight women (36, 37). Here we assessed whether conditions leading to immediately corrected *vs.* permanently altered glycemia, due to well- or ill-functioning of insulin signaling, respectively, are accompanied by altered blood endocannabinoid levels. Healthy volunteers fasted for 12 h and then receiving a meal inducing a strong increase in insulinemia, exhibited blood anandamide levels significantly lower than preprandial levels. In contrast, overweight patients with hyperglycemia caused by type 2 diabetes exhibited blood endocannabinoid levels significantly higher than those of age- and BMI-matched normoglycemic volunteers. These findings might suggest that normal regulation of blood endocannabinoid levels is disrupted in hyperglycemic patients, perhaps in the same way as we have observed here in β-cells grown in high glucose. The fact that, because of ethical reasons, we could not include untreated type 2 diabetes patients is a limitation of this experiment. However, because we wanted to investigate whether permanent as opposed to transient hyperglycemia causes different changes in endocannabinoid levels, we included patients under randomized treatments to avoid biasing of results from the type of treatment.

In conclusion, we have shown that endocannabinoid/CB_1 signaling: 1) participates in adipocyte differentiation and lipid accumulation, and remains activated in mature adipocytes, where it causes depression of adiponectin expression; 2) stimulates insulin secretion in RIN-m5F β-cells and, in turn, is stimulated by insulin under conditions mimicking hyperglycemia; and 3) is overactive in the adipose tissue and pancreas of mice with DIO, a condition similar to human obesity, and in the visceral adipose tissue of obese patients. These findings provide further evidence to the hypothesis that endocannabinoids play a direct role in the peripheral control of energy homeostasis.

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