The Role of Adipocyte Insulin Resistance in the Pathogenesis of Obesity-Related Elevations in Endocannabinoids

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OBJECTIVE—Obesity is associated with an overactive endocannabinoid (EC) system. The mechanisms responsible for increased ECs in obese individuals are poorly understood. Therefore, we examined the role of adipocyte insulin resistance in intracellular EC metabolism.

METHODS—We used 3T3-L1 adipocytes and diet-induced obese (DIO) mice to examine the role of obesity and insulin resistance in the regulation and/or dysregulation of intracellular ECs.

RESULTS—For the first time, we provide evidence that insulin is a major regulator of EC metabolism. Insulin treatment reduced intracellular ECs (2-arachidonylglycerol [2-AG] and anandamide [AEA]) in 3T3-L1 adipocytes. This corresponded with insulin-sensitive expression changes in enzymes of EC metabolism. In insulin-resistant adipocytes, patterns of insulin-induced enzyme expression were disturbed in a manner consistent with elevated EC synthesis and reduced EC degradation. Expression profiling of adipocytes from DIO mice largely recapitulated in vitro changes, suggesting that insulin resistance affects the EC system in vivo. In mice, expression changes of EC synthesis and degradation enzymes were accompanied by increased plasma EC concentrations (2-AG and AEA) and elevated adipose tissue 2-AG.

CONCLUSIONS—Our findings suggest that insulin-resistant adipocytes fail to regulate EC metabolism and decrease intracellular EC levels in response to insulin stimulation. These novel observations offer a mechanism whereby obese insulin-resistant individuals exhibit increased concentrations of ECs. ***Diabetes*** **57:1262–1268, 2008**

Growing evidence suggests that the endocannabinoid (EC) system plays an important role in obesity and metabolic disease. ECs are endogenous lipid-derived molecules that act similarly to the active ingredient in marijuana, Δ^9-tetrahydrocannabinol, to induce food intake. Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are two well-characterized ECs. These molecules are synthesized from precursors in phospholipid membrane by N-arachidonoylphosphotitylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL)-α, while fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) degrade EC substrates. FAAH degrades both AEA and 2-AG (1,2), while MAGL acts on 2-AG (3). ECs are highly regulated in the brain and induce food consumption through activation of the cannabinoid 1 receptors (CB_1Rs) in the hypothalamus (4–6).

Antagonizing CB_1R with the pharmacological agent rimonabant decreases body weight and improves multiple aspects of metabolic disease (i.e., insulin resistance and serum lipids) in overweight and obese patients (7–9). In rodents, rimonabant elicits its initial effects on body weight primarily through a dramatic reduction in food consumption in the initial days of treatment (10), consistent with the EC system's role in the central nervous system. Nevertheless, evidence suggests an additional mechanism independent of food intake by which CB_1R blockade regulates metabolism directly in peripheral tissues (11). Mechanistically, these effects have been explained by the EC system's influence on adiponectin expression (12), liver and adipose lipogenesis (13,14), and adipose tissue energy expenditure and fat oxidation (15).

Adipose tissue is emerging as a key organ involved in the EC system (16–18). Obese humans have elevated circulating and adipose tissue ECs and reduced adipose tissue FAAH expression (17). Overall, little is known about the mechanism by which ECs are elevated in obesity. Here, we evaluated the role of insulin in EC regulation. We demonstrate that insulin regulates intracellular EC levels in 3T3-L1 adipocytes. This regulation corresponds to temporary changes in EC metabolizing enzyme expression consistent with decreased EC synthesis and increased EC degradation. Furthermore, we demonstrate that this regulation is lost in insulin-resistant adipocytes. Differences seen in vivo between lean and obese mice mimic the gene expression patterns of insulin-sensitive versus insulin-resistant adipocytes, suggesting a mechanistic link between insulin resistance and obesity-related increases in ECs.

RESEARCH DESIGN AND METHODS

Cell culture. The mouse fibroblast cell line 3T3-L1 (ATTC, Manassas, VA) was maintained and differentiated as previously described (19). Briefly, differentiation was induced in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 5 μg/ml insulin, 0.25 μmol/l dexamethasone, and 0.5 mmol/l 3-IBMX (isobutyl-1-methylxanthine). After 3 days, insulin, dexamethasone, and IBMX were removed, and cells were maintained in 10% FBS media until the day of experiments (days 7–8). To induce insulin resistance in adipocytes, media was supplemented with 100 nmol/l insulin or vehicle (pH-equivalent distilled water) control from day 3 to days 7–8. All cells were subjected to the same manipulation, and media was changed every 2 days with fresh treatment. Similarly, for shorter duration

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2-AG, 2-arachidonylglycerol; AEA, anandamide; CB_1R, cannabinoid 1 receptor; DAGL, diacylglycerol lipase; EC, endocannabinoid; FAAH, fatty acid amide hydrolase; FBS, fetal bovine serum; MAGL, monoacylglycerol lipase; NAPE-PLD, N-arachidonoylphosphotitylethanolamine phospholipase D.

insulin treatments, 10% FBS media was supplemented with insulin or vehicle and cells where treated for the time described without change of media (2 h–2 days).

Real-time PCR. RNA was isolated and quantified from epididymal fat and cell lysates as previously described (20). cDNA was synthesized from 1 μg RNA using Sprint Powerstrips (Clontech, Mountainview, CA). Taqman real-time quantitative PCR was performed on an ABI 7700 Sequence Detector. Each amplification was performed in triplicate in a 10-μl reaction mixture on a 384-well plate. The reaction mixture included 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nmol/l forward primer and reverse primer, and 250 nmol/l Taqman probe. The primers and probes were purchased from Applied Biosystems Assays on Demand (CB_1R, FAAH, and NAPE-PLD). The primers and probes for MAGL and DAGL-α are as follows: MAGL, F-TGGCCCCAAATAGCTCTTCTC, R-TGCTGTGGGTGACCA AACTC, probe CCCCTCCCACCCTGC; DAGL-α, F-AGAGTTTGTGACTGCT GTGGTT, R-GCTGGGAAAGGCCAATCCT, probe CTGGGCAAAGACCTTG. Data were analyzed using the comparative critical threshold method (21), with the amount of target gene normalized to the endogenous control gene, β2-microglobulin. Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ relative to control (21).

EC extraction and quantification. ECs were extracted from cells and tissues based on previously described methods (22) with modifications. Briefly, samples were homogenized in a solution of Tris buffer (50 mmol/l, pH 8.0), methanol, and chloroform (1:1:2) with 0.1 mmol/l phenylmethylsulfonyl fluoride (FAAH inhibitor). Deuterated internal standards (1 nmol/l), d8-AEA and d8–2-AG (Cayman Chemical, Ann Arbor, MI), were added to the chloroform before extraction. Homogenate was spun at 4°C for 15 min, and the chloroform layer was removed. Homogenization and centrifugation were repeated. Extractions were combined, dried down using a vacuum concentrator (Thermo SPD121P; ThermoScientific, Waltham MA), and frozen at −80°C until further processing. Protein concentration of each extracted sample was quantified using the bicinchoninic assay method (Pierce Biotechnology, Rockford, IL).

For analysis of ECs, samples were reconstituted in 50 μl ethanol before analysis, of which 10-μl aliquots were used for two separate quantitative liquid chromatographic/mass spectrometric analyses using a Waters Acquity UPLC system (Waters, Milford, MA). Liquid chromatographic separation of ECs was achieved by using a Waters SunFire C_{18} 3.5-μm (2.1 × 30 mm) column maintained at ambient temperature. The aqueous mobile phase A consisted of 5 mm ammonium acetate, and the organic mobile phase B was methanol. The flow rate was set to 0.250 ml/min; initial conditions were 80% A and 20% B held for 1 min and then linearly ramped to 1% A/99% B and held for 4 min; after 6 s, the system was returned to initial conditions. A Quattro Premier XE triple quadruple mass spectrometer (Waters) was operated under positive electrospray ionization mode and multiple reaction monitoring set to monitor ions *m/z* 379 for 2-AG, 387 for 2-AG(d8), 348 for AEA, and 356 for AEA(d8). Source conditions were as follows: capillary 3.3 kV, source temperature 110°C, desolvation temperature 300°C, and nitrogen drying gas 500 l/h. Calibration curves were generated using synthetic anandamide, 2-AG, and their deuterated analogs (Cayman Chemical, Ann Arbor, MI). The amounts of AEA and 2-AG in the samples were determined by using Micromass QuanLynx, version 4.1, software. Values are expressed as fentomol or picomol per mg protein.

Diet-induced obesity. All experiments were approved by the Novartis Institutes for Biomedical Research animal care and use committee. Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age. For acclimation, animals were caged individually for 2 weeks before experiments, with free access to water and normal chow. At 6 weeks of age, mice were divided into two groups ($n = 8$ per group) and fed with regular chow or a high-fat diet (60% lard) for 9 weeks (D12492, Research Diets, New Brunswick, NJ). On the day of animal sacrifice, free-fed (non-

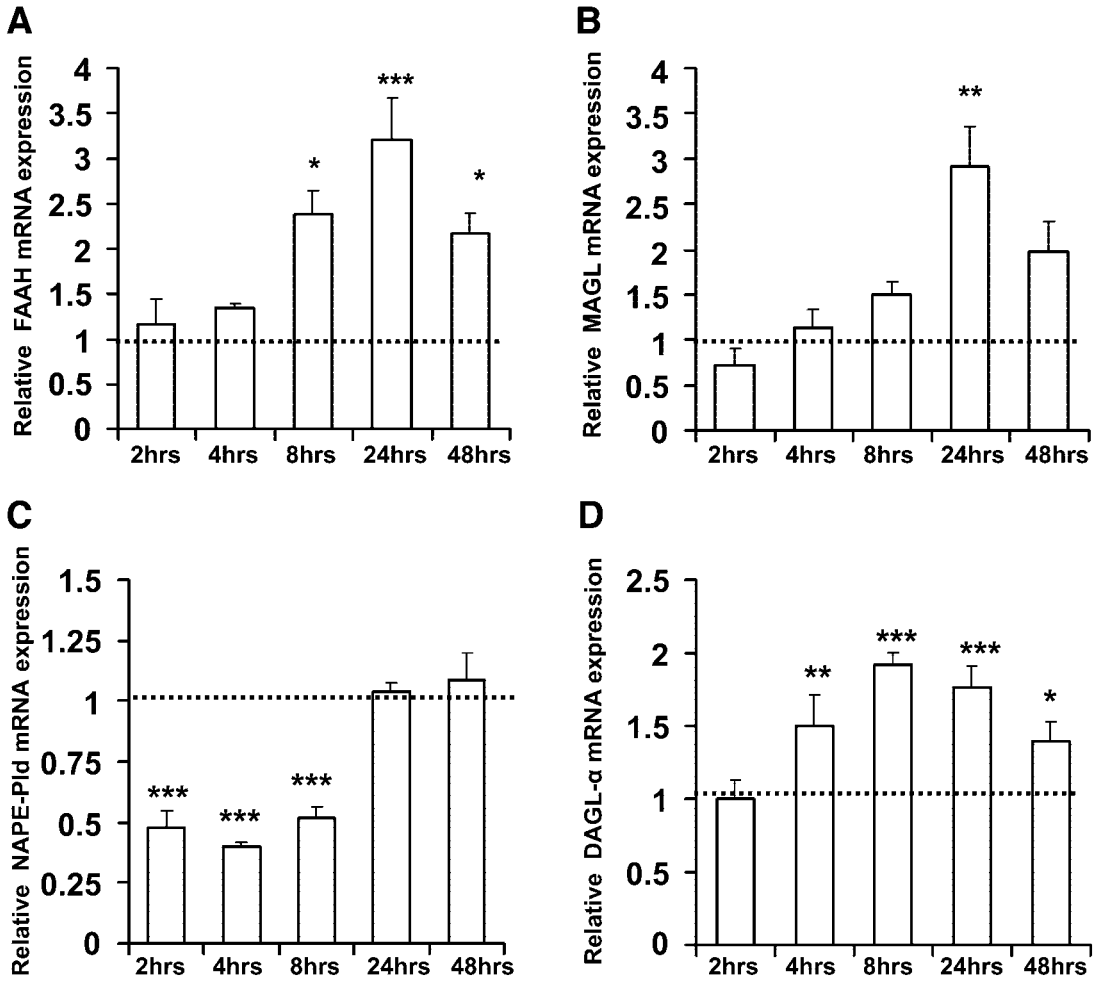

FIG. 1. Effect of insulin on the expression of enzymes involved in EC synthesis and degradation. Differentiated adipocytes were treated with insulin (100 nmol/l). Data are expressed relative to control (untreated) cells at each time point. *A*: FAAH mRNA expression. *B*: MAGL mRNA expression. *C*: NAPE-PLD mRNA expression. *D*: DAGL-α mRNA expression. Data are means ± SEM; $n = 3$ individual experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to untreated control.

fasted) mice were killed with CO_2, plasma was collected by cardiac puncture, and epididymal adipose tissue was extracted, immediately frozen in liquid nitrogen, and stored in a −80°C freezer until analyses.

Statistical analysis. Data are shown as means ± SEM. Data were analyzed by one-way ANOVA, and the Student Newman-Keuls test was used for post hoc analysis. Statistical analyses were conducted with GraphPad Prism4 (San Diego, CA) with an α level of 0.05.

RESULTS

Insulin regulates intracellular EC concentrations in adipocytes. We hypothesized that insulin resistance may be involved in the pathogenesis of obesity-related elevations in ECs, which predicts a direct effect of insulin on ECs. We observed that 24-h insulin treatment reduces intracellular AEA ($P < 0.05$) and 2-AG ($P < 0.05$) (Table 1) in 3T3-L1 adipocytes. To further understand the impact of insulin, we examined how this hormone affected the expression of enzymes involved in EC metabolism. Insulin increased the expression of the EC-degrading enzyme FAAH in a time-dependent manner (Fig. 1*A*). MAGL, which degrades 2-AG, increased with insulin treatment in a similar pattern (Fig. 1*B*). Expression of AEA-synthesizing enzyme, NAPE-PLD, was rapidly (2 h) reduced following insulin treatment (Fig. 1*C*). In contrast, 2-AG–synthesizing enzyme, DAGL-α (Fig. 1*D*), increased with insulin treatment. Insulin did not regulate expression of CB_1R (data not shown).

TABLE 1
Effect of insulin on adipocyte EC levels

	Control	Insulin
AEA (fmol/mg)	22.0 ± 3.5	13.3 ± 2.1*
2-AG (pmol/mg)	30.7 ± 3.6	22.6 ± 3.6*

Data are means ± SEM. Intracellular ECs were quantified in 3T3-L1 adipocytes following 24-h insulin treatment. $n = 4$ independent experiments. *$P < 0.05$.

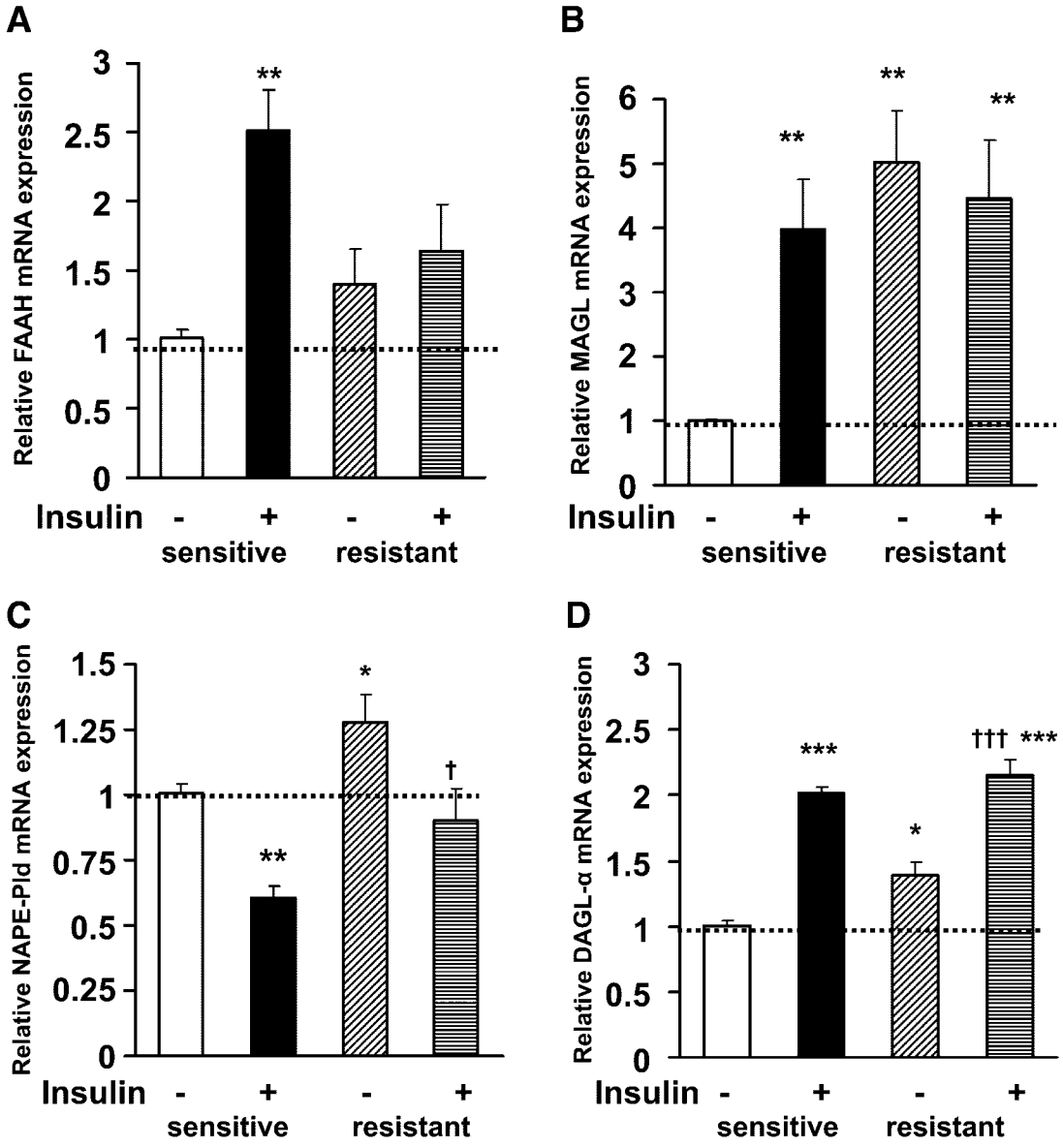

FIG. 2. Effect of insulin resistance on the ability of adipocytes to regulate the enzymes involved in EC synthesis and degradation. *A*: FAAH mRNA expression after 24 h treatment. *B*: MAGL mRNA expression after 24 h treatment. *C*: NAPE-PLD mRNA expression after 8 h treatment. *D*: DAGL-α mRNA expression after 24 h treatment. □, insulin-sensitive control; ■, insulin sensitive and insulin treated; ▨, insulin-resistant control; ▤, insulin resistant and insulin treated. Data are means ± SEM; $n = 3$ individual experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to insulin-sensitive control; †$P < 0.05$ relative to insulin-resistant control.

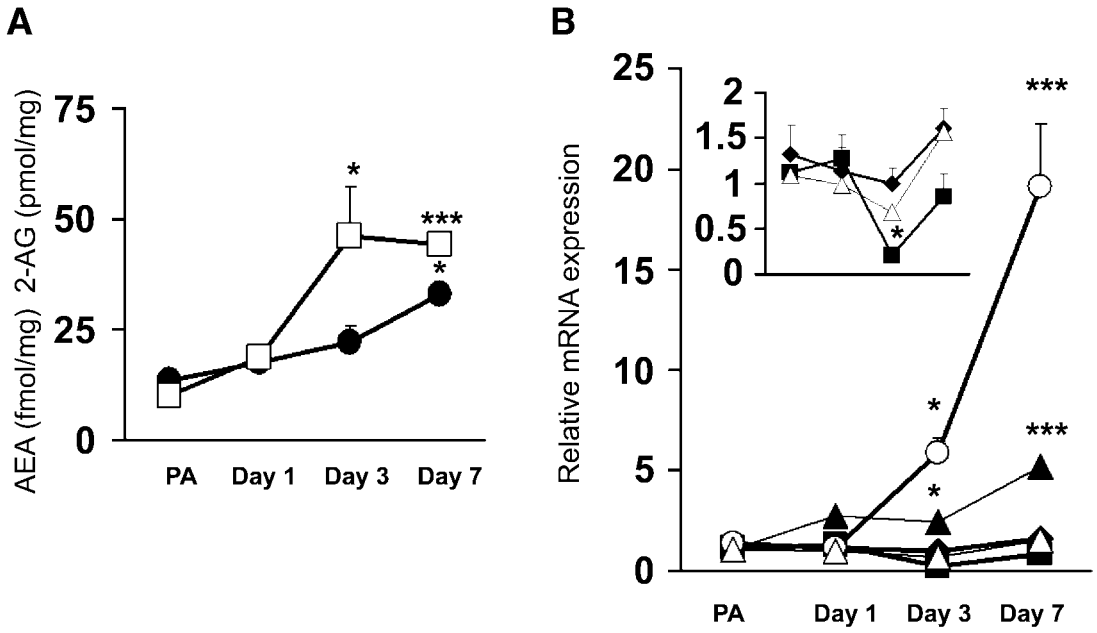

FIG. 3. Quantification of intracellular ECs and the enzymes needed for synthesis and degradation during adipocyte differentiation. *A*: Intracellular AEA (●) and 2-AG (□) during differentiation. *B*: mRNA expression of MAGL (○), NAPE-Pld (▲), DAGL-α (◇), CB_1R (◆), and FAAH (■) during adipocyte differentiation. For clarity, genes not upregulated by differentiation (CB_1R, FAAH, and DAGL-α) are shown in the inset. Data are means ± SEM; $n = 3$ individual experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Insulin-resistant adipocytes are unable to regulate EC degradation. We observed that gene expression of EC system components change in response to insulin, and these changes correlated with reduced intracellular EC levels. We hypothesized that a state of insulin resistance could alter these relationships, suggesting a further mechanism for EC system dysregulation in obesity. To test this hypothesis, we made 3T3-L1 adipocytes insulin resistant through chronic exposure to insulin as previously described (23).

Next, we examined whether insulin resistance affected insulin-dependent regulation of the EC system. Expression assays were performed at 8 h (DAGL-α and NAPE-PLD) or 24 h (FAAH and MAGL) when significant regulation was previously observed (Fig. 1). Insulin-resistant cells were unable to increase FAAH gene expression in response to insulin (Fig. 2*A*). MAGL also became insulin insensitive (Fig. 2*B*); basal MAGL in insulin-resistant cells, however, was observed at levels similar to that in insulin-sensitive cells treated with insulin. However, both NAPE-PLD and DAGL-α expression were increased in resistant cells ($P < 0.05$). These enzymes, in contrast, remained responsive to insulin (Fig. 2*C* and 2*D*). Significantly, insulin was unable to reduce intracellular ECs in insulin-resistant adipocytes (2-AG, control 15.7 ± 1.0 and insulin 15.1 ± 1.2 pmol/mg; AEA, control 7.6 ± 1.3 and insulin 5.38 ± 1.4 fmol/mg). Collectively, these data show that insulin resistance alters insulin-induced EC reduction in adipocytes by altering insulin-sensitive expression of EC system enzymes.

Regulation of ECs during adipocyte differentiation. To further explore EC regulation in adipose tissue and the changes in intracellular EC levels that may occur with obesity (i.e., during adipogenesis), we examined whether gene expression of EC system components and intracellular ECs concentrations changed during adipocyte differentiation (Fig. 3). Both 2-AG and AEA were present in preadipocytes and increased during differentiation (Fig. 3*A*). Expression of CB_1R and DAGL-α remained constant during adipocyte differentiation (Fig. 3*B*). There was a moderate but significant reduction in FAAH expression ($P < 0.05$) on day 3 before removal of the adipogenic media (Fig. 3*B*). Both NAPE-PLD (5.2 ± 0.7-fold) and MAGL (19.1 ± 3.0-fold) were upregulated during differentiation (Fig. 3*B*). As a positive control for gene expression, we observed that peroxisome proliferator–activated receptor-γ was upregulated 36.9 ± 5.7-fold during differentiation.

Obesity-related alterations in adipose tissue expression of EC-metabolizing enzymes mimic patterns observed in insulin-resistant adipocytes. Finally, we examined lean and DIO mice to determine whether our observations in insulin-resistant adipocytes in vitro were consistent with an altered expression of components of the EC system in vivo. Previous studies have demonstrated adipose tissue insulin resistance in C57 Bl6 mice on a high-fat diet as early as 3 weeks into the diet (24). Similar to observations in insulin-resistant adipocytes (Fig. 2), adipose tissue from obese mice showed reduced FAAH expression ($P < 0.01$) (Fig. 4*A*) and significant increases in MAGL ($P < 0.5$ (Fig. 4*B*) and DAGLα ($P < 0.01$) (Fig. 4*D*). No differences were observed for adipose tissue NAPE-PLD (Fig. 4*C*) or CB_1R expression (data not shown) with obesity.

In addition, plasma AEA and 2-AG concentrations were significantly elevated ($P < 0.001$) in DIO mice compared with those in lean mice (Table 2). Adipose tissue 2-AG levels were increased ($P = 0.05$), whereas AEA levels were unchanged (Table 2). These data suggest that transcriptional regulation of EC system components in adipocyte is consistently altered in settings of insulin resistance.

DISCUSSION

Obesity is considered a condition of overactivation of the EC system, a physiological state exploited by the pharmacological use of CB_1R antagonists in the treatment of obesity (17). Although the EC system has traditionally

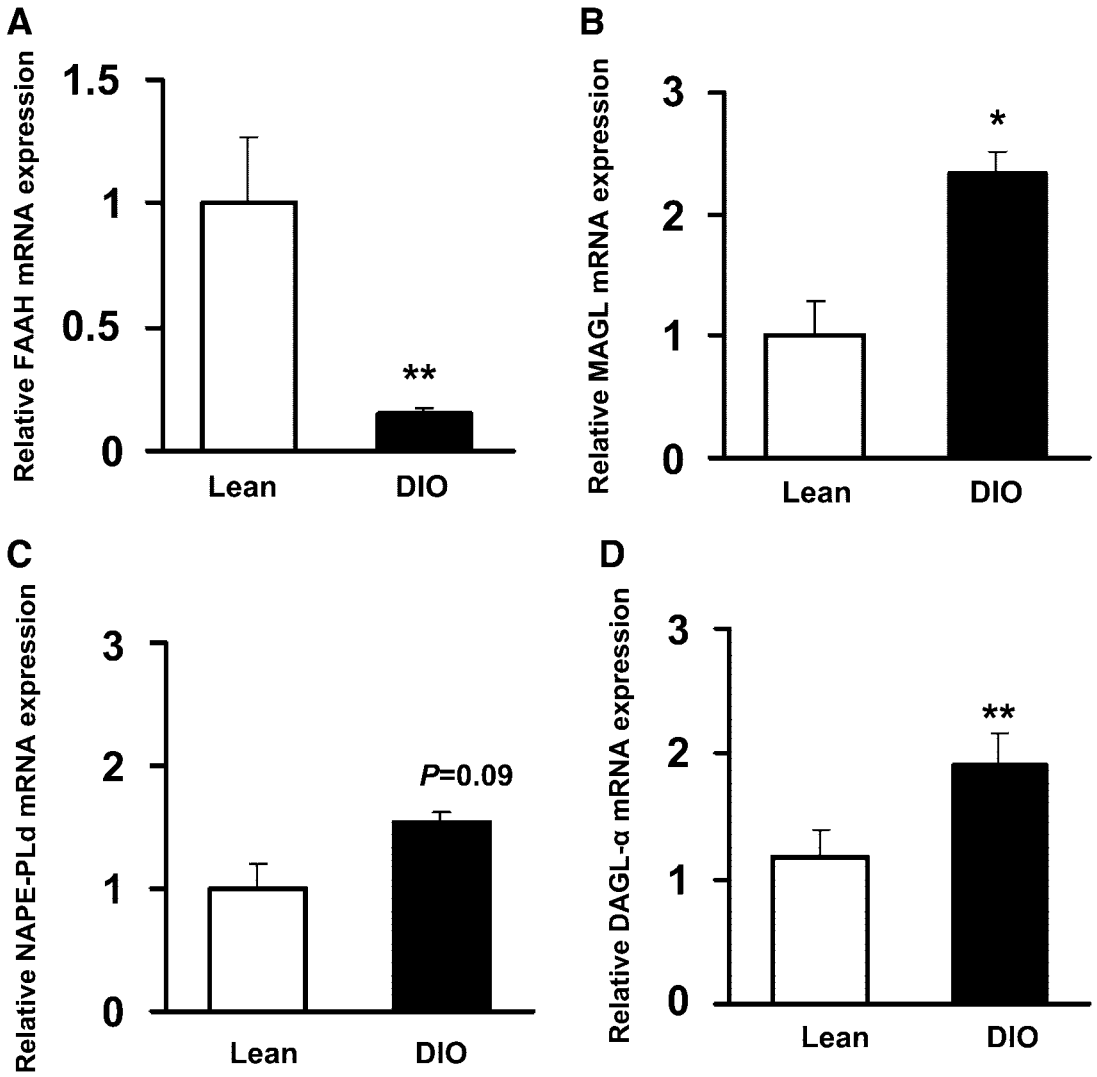

FIG. 4. Effect of obesity on the expression of enzymes involved in EC synthesis and degradation. *A*: FAAH mRNA expression. *B* : MAGL mRNA expression. *C*: NAPE-PLD mRNA expression. *D*: DAGL-α mRNA expression. □, lean; ■, DIO. Data are means ± SEM; $n = 8$ mice/group. *$P < 0.05$; **$P < 0.01$.

been considered a brain-specific regulatory pathway, evidence suggests that it plays a role in peripheral tissue and, in particular, adipose tissue (16,17,25). This concept is supported by a growing body of literature demonstrating that signaling networks acting in the brain are also relevant in adipose tissue (26). In this study, we present data suggesting adipocyte insulin resistance contributes to a dysfunctional EC system in the obese state. We propose a dual role of the adipocyte in EC metabolism involving both local regulation of intracellular adipose tissue ECs and also regulation of circulating ECs through membrane-bound EC-degrading enzyme FAAH. Most importantly, we propose that both of these systems are dysregulated by insulin resistance, contributing to the observed obesity-related overactivation of the EC system.

For the first time, we demonstrate that insulin is a potentially significant regulator of EC metabolism in normal adipocytes. Insulin induced multiple changes that lead to reduced intracellular EC levels, likely through increasing expression of MAGL and FAAH, while reducing NAPE-

TABLE 2
Effect of obesity on circulating and adipose tissue ECs

	Lean	DIO
Body weight (g)	29.5 ± 0.9	49.08 ± 1.2*
Insulin (pmol/l)	154.3 ± 17.0	213.3 ± 24.1 ($P = 0.060$)
Glucose (mg/dl)	171.0 ± 6.1	171.6 ± 4.2
Plasma AEA (pmol/ml)	471.7 ± 90.0	1260.8 ± 260.0*
Plasma 2-AG (nmol/ml)	40.0 ± 9.0	116.0 ± 12.2*
Adipose tissue AEA (fmol/mg)	63.5 ± 13.4	78.2 ± 11.7
Adipose tissue 2-AG (pmol/mg)	17.6 ± 2.3	28.3 ± 1.9 ($P = 0.050$)

Data are means ± SEM. $n = 8$ mice per group. *$P < 0.05$.

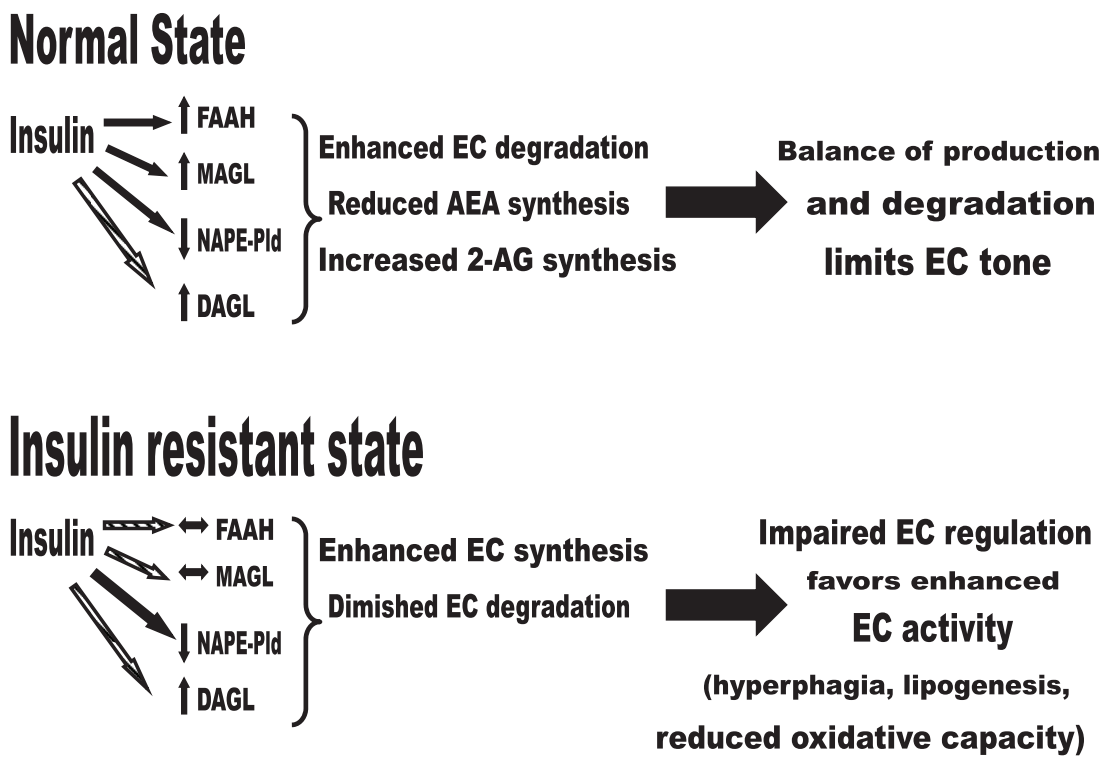

FIG. 5. Summary of the effects of insulin on EC metabolism in the normal vs. the insulin-resistant state. Closed arrows, promoting reduced intracellular ECs; striped arrow, promoting equal or elevated intracellular ECs.

Pld. An exception to this, however, is the 2-AG–synthesizing enzyme DAGL-α, which is increased with insulin treatment. Ultimately, however, intracellular 2-AG was reduced, potentially explained by a threefold increase in degrading enzyme MAGL.

Because of the clear role of insulin in reducing intracellular ECs and regulating transcription of the genes involved in EC metabolism, we hypothesized that insulin resistance would lead to altered regulation of intracellular ECs. In our adipocyte model, insulin-mediated induction of both FAAH and MAGL is abrogated, while DAGL-α induction remains insulin sensitive and high in the basal insulin-resistant state. This implies that in conditions of hyperinsulinemia, chronically elevated insulin levels may enhance 2-AG production while being unable to increase 2-AG degradation. Collectively, our observations suggest that during obesity, altered gene expression in insulin-resistant adipose tissue may promote increased EC synthesis and reduced EC degradation.

Consistent with this notion, we observed increased adipose 2-AG levels between lean and obese mice. Expression patterns in the adipose tissue of obese mice were similar to insulin-resistant adipocytes (i.e., elevated MAGL and DAGL-α, and reduced FAAH), suggesting that the 3T3-L1 model can be used to further deduce mechanisms of EC dysregulation in obesity. Our findings suggest that adipocyte FAAH levels can be considered insulin dependent, suggesting a mechanism as to why adipose FAAH expression is reduced in obese individuals (17). FAAH is localized on extracellular membranes and has been suggested to act on circulating ECs (16). In light of our data, this could explain why circulating EC levels are negatively correlated with insulin sensitivity in humans (27). Consistent with data in humans, we observed elevated circulating AEA and 2-AG but only elevated 2-AG in adipose tissue of obese mice (17). The reason AEA is unchanged in adipose tissue in unknown; however, this provides further evidence that adipose tissue may be involved in EC metabolism both by regulating the degradation of circulating ECs derived from other sourcesand by local synthesis and/or degradation, as circulating level are altered with obesity. Interestingly, the physiological consequences in adipose-specific overproduction of EC include reduced adiponectin expression (12), enhanced lipogenesis (13,14), and reduced adipose tissue energy expenditure and fat oxidation (15), all of which are processes proposed to be dysregulated with insulin resistance and obesity.

There is a growing body of literature suggesting a significant role for ECs in peripheral tissues including muscle and liver. Data from human studies using the CB_1R antagonist rimonanbant demonstrates that ~50% of the beneficial effects of CB_1R blockade on HDLs, adiponectin, and triacyglycerides are independent of weight loss (7,8). Clearly, there is much to be explored with regard to the role of the EC system in peripheral tissues, but emerging evidence suggests it can no longer be considered solely a centrally acting system.

In conclusion, our data implicate adipocyte insulin resistance as a contributing factor in obesity-related EC system aberrations. Insulin-induced regulatory patterns of the EC system are lost in insulin-resistant adipocytes, resulting in an inability to reduce intracellular ECs and thereby enhancing EC signaling (summarized in Fig. 5). Our study predicts that in vivo loss and gain-of-function models could reveal determinative roles for FAAH and DAGL-α in the EC system; however, establishing a comprehensive hierarchy of EC system regulation will require tissue-specific modulation of these enzymes in at least brain and fat. Collectively, we establish insulin resistance as an element of EC system dysregulation in obesity,

providing novel investigative avenues in the therapy of metabolic disorders.

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