

**The role of adipocyte insulin resistance in the pathogenesis of obesity-related elevations in endocannabinoids**

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## ABSTRACT

*Objective:* Obesity is associated with an over-active endocannabinoid system (ECS). The mechanisms responsible for increased endocannabinoids (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-arachidonylethanolamide (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 ECS *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.

*Conclusion:* 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.

Growing evidence suggests the endocannabinoid system (ECS) plays an important role in obesity and metabolic disease. Endocannabinoids (EC) are endogenous lipid-derived molecules that act similarly to the active ingredient in marijuana,  $\Delta^9$ -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-arachidonoylphosphotylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase  $\alpha$  (DAGL- $\alpha$ ), 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<sub>1</sub>R) in the hypothalamus (4-6).

Antagonizing CB<sub>1</sub>R with the pharmacological agent rimonabant decreases body weight and improves multiple aspects of metabolic disease (i.e. insulin resistance, 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 ECS's role in the central nervous system. Nevertheless, evidence suggests an additional mechanism independent of food intake by which CB<sub>1</sub>R blockade regulates metabolism directly in peripheral tissues (11). Mechanistically, these effects have been explained by the ECS'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 ECS (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 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 (DMEM) containing 10% fetal bovine serum (FBS), 5  $\mu$ g/ml of insulin, 0.25  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After 3 days, insulin, dexamethasone, and IBMX were removed and cells were maintained in 10% FBS media until the day of experiments (Day 7-8). To induce insulin resistance in adipocytes, media was supplemented with 100 nM insulin or vehicle (pH-equivalent distilled water) control from Day 3 until Day 7-8. All cells were subjected to the same manipulation and media was changed every 2 days with fresh treatment. Similarly, for shorter duration insulin treatments, 10% FBS media was supplemented with insulin or vehicle and cells were treated for the time described without change of media (2 hours to 2 days).

**Realtime PCR.** RNA was isolated and quantified from epididymal fat and cell lysates as previously described (20). Complementary DNA (cDNA) was synthesized from 1 ug of 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- $\mu$ l reaction mixture on a 384-well plate. The reaction mixture included 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM of forward primer and reverse primer, and 250 nM of Taqman probe. The primers and probes were purchased from Applied Biosystems “Assays on Demand” (CB<sub>1</sub>R, FAAH, and NAPE-PLd). The primers and probes for MAGL and DAGL- $\alpha$  are as follows: MAGL: F-TGGCCCCAAATAGCTCTTCTC, R-TGCTGTGGGTGACCAAACCTC, probe CCCCTCCCACCCTGC; DAGL- $\alpha$ : F-AGAGTTTGTGACTGCTGTGGTT, 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, beta 2 microglobulin. Relative gene expression was calculated by  $2^{-\Delta\Delta C_t}$  relative to control (21).

**Endocannabinoid extraction and quantification.** Endocannabinoids 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 mM, pH 8.0), methanol, and chloroform (1:1:2) with 0.1 mM phenylmethylsulfonyl fluoride (FAAH inhibitor). Deuterated internal standards (1nM), d8-AEA and d8-2-AG (Cayman Chemical, Ann Arbor, Michigan), were added to the chloroform prior to extraction. Homogenate was spun at 4°C for 15 minutes and the chloroform layer

was removed. Homogenization and centrifugation was 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 Inc., Rockford, IL).

For analysis of ECs samples were reconstituted in 50  $\mu$ L of ethanol prior to analysis, of which 10  $\mu$ L aliquots were used for two separate quantitative liquid chromatographic/mass spectrometric analyses using a Waters Acquity UPLC system (Waters Corp., Milford, MA). Liquid chromatographic separation of endocannabinoids was achieved by using a Waters SunFire C<sub>18</sub> 3.5  $\mu$ m (2.1 x 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 one minute, then linearly ramped to 1% A / 99% B and held for four minutes, after six seconds the system was returned to initial conditions. A Quattro Premier XE triple quadrupole mass spectrometer (Waters Corp., Milford, MA) 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, nitrogen drying gas at 500 L/hr. 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 V4.1 software. Values are expressed as fmol or pmol per mg protein.

**Animals - Diet-induced obesity.** All experiments were approved by the Novartis Institutes for Biomedical Research, Inc. 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 prior to experiments, with free access to water and normal chow. At 6 weeks of age, mice were divided into 2 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-fasted) mice were euthanized with CO<sub>2</sub>, plasma was collected by cardiac puncture, and epididymal adipose tissue was extracted, immediately frozen in liquid nitrogen, and stored in -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 posthoc analysis. Statistical analyses were conducted with GraphPad Prism4 (San Diego, CA) with an  $\alpha$  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: this predicts a direct effect of insulin on ECs. We observed that 24-hr 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 1A). MAGL, which degrades 2-AG, increased with insulin treatment in a similar pattern (Fig. 1B). Expression of AEA-synthesizing

enzyme, NAPE-Pld, was rapidly (2 hrs) reduced following insulin treatment (Fig. 1C). In contrast, 2-AG synthesizing enzyme, DAGL- $\alpha$  (Fig. 1D) increased with insulin treatment. Insulin did not regulate expression of CB<sub>1</sub>R (data not shown).

**Insulin resistant adipocytes are unable to regulate EC degradation.** We observed that gene expression of ECS 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 ECS 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 ECS. Expression assays were performed at 8 hrs (DAGL- $\alpha$  and NAPE-Pld) or 24 hrs (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. 2A). MAGL also became insulin-insensitive (Fig. 2B); basal MAGL in insulin resistant cells, however, was observed at levels similar to insulin sensitive cells treated with insulin. However, both NAPE-Pld and DAGL- $\alpha$  expression were increased in resistant cells ( $P < 0.05$ ). These enzymes, in contrast, remained responsive to insulin (Fig. 2C and 2D). Significantly, insulin was unable to reduce intracellular ECs in insulin resistant adipocytes (2-AG (pmol/mg); control  $15.7 \pm 1.0$ , insulin  $15.1 \pm 1.2$ ; AEA (fmol/mg) control  $7.6 \pm 1.3$ , insulin  $5.38 \pm 1.4$ ). Collectively, these data show that insulin resistance alters insulin-induced EC reduction in adipocytes by altering insulin-sensitive expression of ECS 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 ECS 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. 3A). Expression of CB<sub>1</sub>R and DAGL- $\alpha$  remained constant during adipocyte differentiation (Fig.3B). There was a moderate but significant reduction in FAAH expression ( $P<0.05$ ) on Day 3, prior to removal of the adipogenic media (Fig. 3B). Both NAPE-Pld ( $5.2 \pm 0.7$ -fold) and MAGL ( $19.1 \pm 3.0$ -fold) were upregulated during differentiation (Fig. 3B). As a positive control for gene expression, we observed that PPAR- $\gamma$  was upregulated  $36.9 \pm 5.7$ -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 diet-induced obese (DIO) mice to determine if our observations in insulin resistant adipocytes *in vitro* were consistent with an altered expression of components of the ECS *in vivo*. Previous studies have demonstrated adipose tissue insulin resistance in C57 Bl6 mice on high fat diet as early as 3 weeks on diet (24). Similar to observations in insulin resistant adipocytes (Figure 2), adipose tissue from obese mice showed reduced FAAH expression ( $P<0.01$ ; Fig. 4A) and significant increases in MAGL ( $P<0.5$ ; Fig. 4B) and DAGL $\alpha$  ( $P<0.01$ ; Fig. 4D). No differences were observed for adipose tissue NAPE-Pld (Fig. 4C) or CB<sub>1</sub>R expression (data not shown) with obesity.

In addition, plasma AEA and 2-AG concentrations were significantly elevated ( $P<0.001$ ) in DIO mice compared to 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 ECS components in adipocyte is consistently altered in settings of insulin resistance.

## DISCUSSION

Obesity is considered a condition of “over-activation” of the ECS, a physiological state that is exploited by the pharmacological use of CB<sub>1</sub>R antagonists in the treatment of obesity (17). Although the ECS has traditionally 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 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 ECS 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 over-activation 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-Pld. An exception to this however, is the 2-AG synthesizing enzyme DAGL- $\alpha$ , which is increased with insulin treatment. Ultimately, however, intracellular 2-AG was reduced, potentially explained by a 3-fold 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- $\alpha$  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, DAGL- $\alpha$ , and reduced FAAH), suggesting 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 is unknown however this provides further evidence that adipose tissue may be involved in EC metabolism by both regulating the degradation of circulating ECs derived from

other sources, as well as 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 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<sub>1</sub>R antagonist, Rimonabant, demonstrates that approximately 50% of the beneficial effects of CB<sub>1</sub>R blockade on high density lipoproteins, adiponectin, and triacylglycerides are weightloss independent(7;8). Clearly, there is much to be explored with regard to the role of the ECS 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 ECS aberrations. Insulin-induced regulatory patterns of the ECS are lost in insulin resistant adipocytes, resulting in an inability to reduce intracellular ECs thereby enhancing EC signaling (summarized in Figure 5). Our study predicts *in vivo* loss and gain-of function models could reveal determinative roles for FAAH and DAGL- $\alpha$  in the ECS, however establishing a comprehensive hierarchy of ECS regulation will require tissue-specific modulation of these enzymes in at least brain and fat. Collectively, we establish insulin resistance as an element of ECS dysregulation in obesity, providing novel investigative avenues in the therapy of metabolic disorders.

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## **TABLE LEGENDS**

**TABLE 1. Effect of insulin on adipocyte EC levels.** Intracellular endocannabinoids were quantified in 3T3-L1 adipocytes following 24-hour insulin treatment. (mean  $\pm$  SEM, n = 4 independent experiments \* $P$ <0.05)

**TABLE 2. Effect of obesity on circulating and adipose tissue ECs.** (mean  $\pm$  SEM, n = 8 mice per group, \* $P$ <0.001)

**Table 1**

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

Table 2

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

## FIGURE LEGENDS

**Figure 1. Effect of insulin on the expression of enzymes involved in EC synthesis and degradation.** Differentiated adipocytes were treated with insulin (100nM). Data is 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- $\alpha$  mRNA expression (mean  $\pm$  SEM, n = 3 individual experiments \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 relative to untreated control)

**Figure 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 24hr treatment **B.** MAGL mRNA expression after 24 hour treatment **C.** NAPE-PLd mRNA expression after 8 hour treatment **D.** DAGL- $\alpha$  mRNA expression after 24hr treatment. (open bars = insulin sensitive control, closed bars = insulin sensitive insulin treated, diagonal stripes = insulin resistant control, horizontal bars = insulin resistant insulin treated; mean  $\pm$  SEM, n = 3 individual experiments \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 relative to insulin sensitive control; †p<0.05 relative to insulin resistant control).

**Figure 3. Quantification of intracellular ECs and the enzymes needed for synthesis and degradation during adipocyte differentiation.** **A.** Intracellular AEA (closed circle) and 2-AG (opened square) during differentiation. **B.** mRNA expression of MAGL (opened circle), NAPE-PLd (closed triangle), DAGL- $\alpha$  (opened triangle), CB<sub>1</sub>R (closed diamond) and FAAH (closed square) during adipocyte differentiation. For clarity, genes which were not up-regulated by differentiation (CB<sub>1</sub>R, FAAH, and DAGL- $\alpha$ ) are shown in the inset. (mean  $\pm$  SEM, n = 3 individual experiments, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001)

**Figure 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- $\alpha$  mRNA expression. (open bars = lean, closed bars = DIO; mean  $\pm$  SEM, n = 8 mice/group, \*p<0.05, \*\*p<0.01).

**Figure 5. Summary of the effects of insulin on EC metabolism in normal versus insulin resistant state.** closed arrows = promoting reduced intracellular ECs, striped arrow = promoting equal or elevated intracellular ECs.

Figure 1

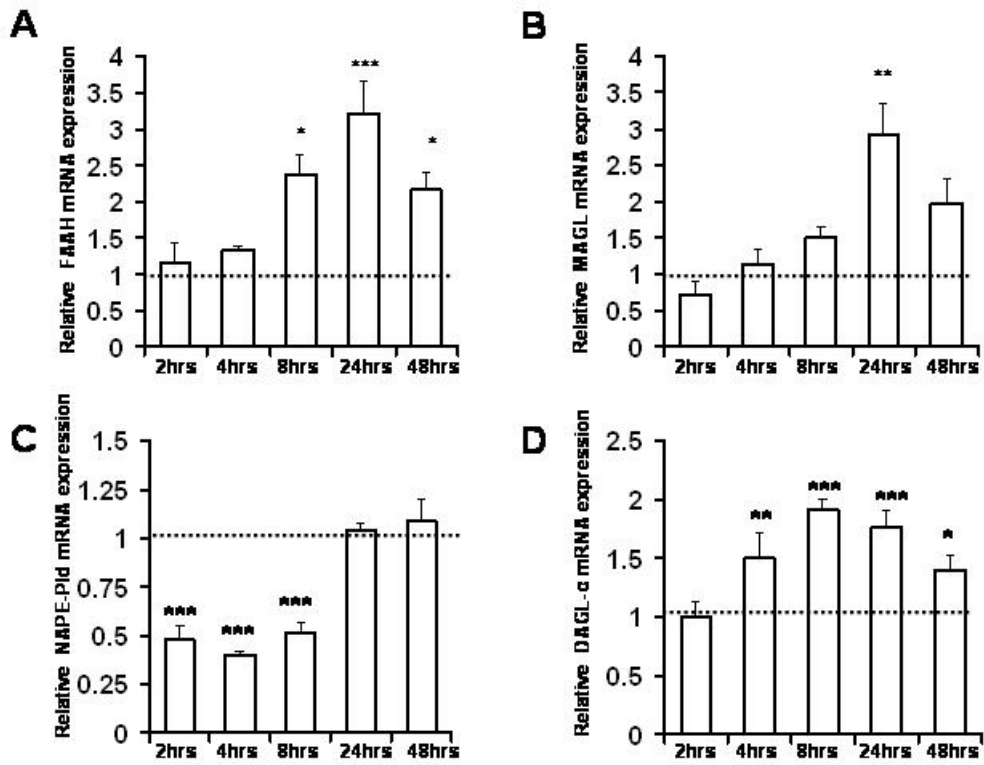


Figure 2

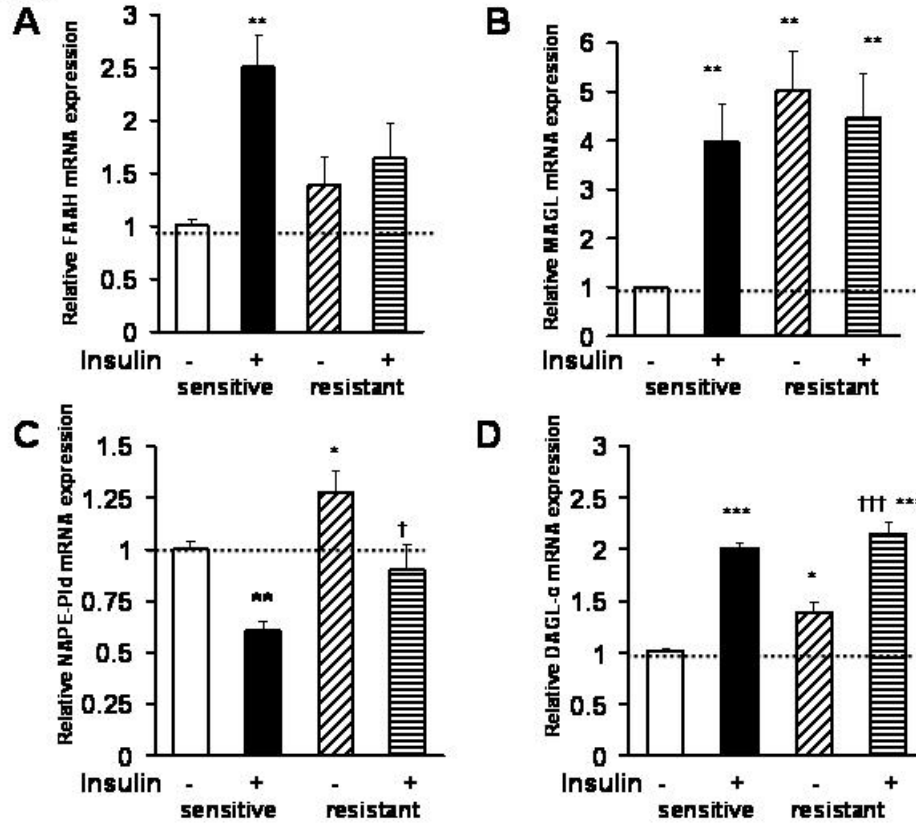


Figure 3

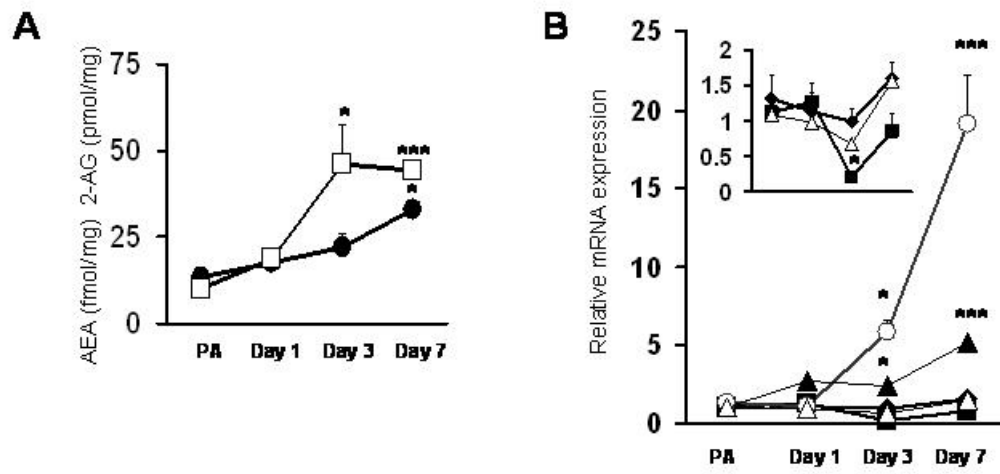


Figure 4

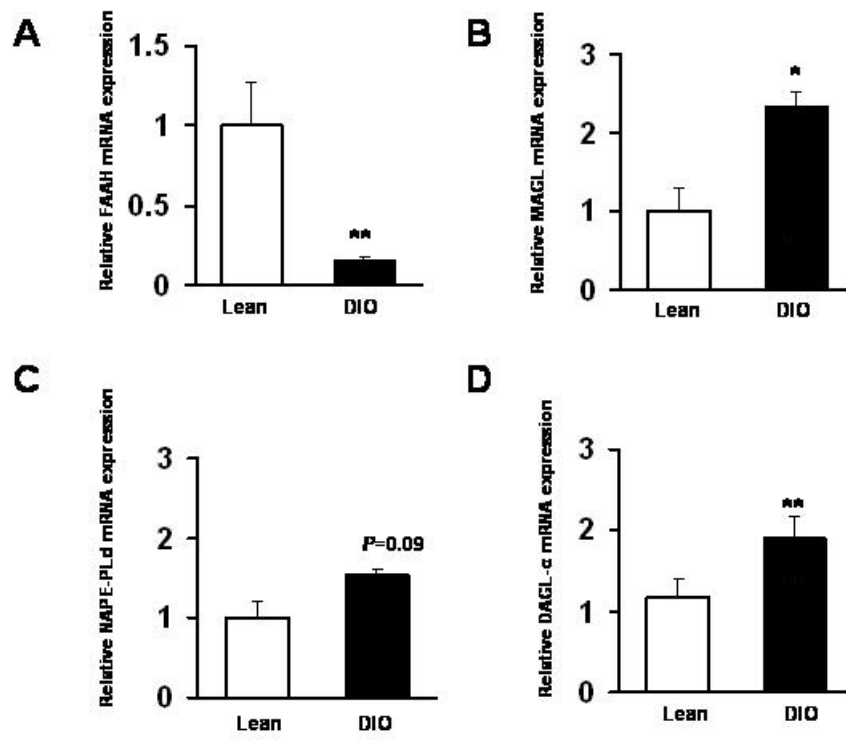
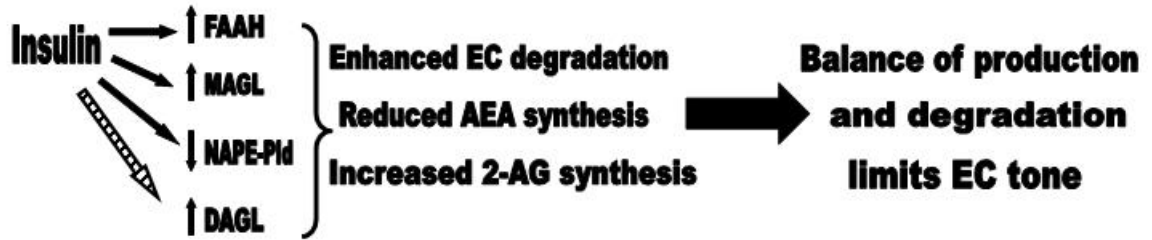


Figure 5

## Normal State



## Insulin resistant state

