# Effects of PPAR-γ and -α agonists on the regulation of malonyl-CoA decarboxylase, acetyl-CoA carboxylase, and AMP-activated protein kinase in OLETF rats

**Thesis by**
**Zheng-Shan Zhao**

**Department of Medical Science**

**The Graduate School, Yonsei University**

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**Directed by Professor Bong-Soo Cha**

**The Doctoral dissertation**

**Submitted to the Department of Medical Science The Graduate School, Yonsei University**

**Zheng-Shan Zhao**

**June 2004**

This certifies that the Doctoral Dissertation
of Zheng-Shan Zhao is approved.

.....
[Thesis Supervisor: Bong-Soo Cha]

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[Thesis Committee Member]

.....
[Thesis Committee Member]

.....
[Thesis Committee Member]

.....
[Thesis Committee Member]

The Graduate School
Yonsei University

June 2004

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By author

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Abstract

# Effects of PPAR-γ and -α agonists on the regulation of malonyl-CoA decarboxylase, acetyl-CoA carboxylase, and AMP-activated protein kinase in OLETF rats

Zheng-Shan Zhao

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Bong-Soo Cha)

It is known that the activities of malonyl-CoA decarboxylase (MCD) and acetyl-CoA carboxylase (ACC) are regulated by AMP-activated protein kinase (AMPK) in rat muscle, heart, and liver in response to exercise. To examine the effects of PPAR-γ and -α agonists on the activities of MCD, ACC, and AMPK and their regulation, rosiglitazone (4 mg/kg/d) and fenofibrate (100 mg/kg/d) were administrated for two weeks in normal and high fat diet fed type 2 diabetic OLETF rats in the prediabetic stage, and MCD, ACC, AMPK activities, and MCD mRNA expression levels were measured. The results suggest that the decrease in body weight in fenofibrate treated

rats in both normal diet and high fat fed groups was mainly due to the decrease in food intake. The increase in fat mass in high fat fed control and rosiglitazone treated rats accounted for the great percents in body weight gains. In muscles of fed normal diet rats treated with either rosiglitazone or fenofibrate, MCD activity increased *2.4-fold* and *1.8-fold* respectively, compared to normal diet control; in muscles of high fat fed rats treated with either rosiglitazone or fenofibrate, MCD activity increased *2.0-fold* and *4.5-fold* respectively, compared to high fat fed control. Both AMPK and ACC activities did not show significant changes either in normal diet or in high fat fed groups. In heart, AMPK activity increased only in fenofibrate treated rats, *2.3-fold* in normal diet and *1.7-fold* in high fat fed groups. AMPK activity in high fat fed rats significantly increased compared to those in normal diet fed control, rosiglitazone, and fenofibrate treated rats. MCD activity increased 30% in normal diet fed rats treated with fenofibrate and decreased 41% in high fat fed rats treated rosiglitazone and fenofibrate, respectively, compared to corresponsive controls. No significant changes in ACC activity in all experimental animals were observed. In liver, AMPK activity decreased 72% in fenofibrate treated rats in high fat fed groups. MCD activity increased *2.7-fold* only in fenofibrate treated rats in normal diet fed group and *1.6-fold* and *2.0-fold* both in rosiglitazone and in fenofibrate treated rats in high fat fed group. ACC activity decreased 38% in rosiglitazone and 87% in fenofribrate treated rats compared to control in high fat fed group. ACC activity in fenofibrate treated rats decreased 79% compared to that of rosiglitazone treated rats in high fat fed group. MCD mRNA expression levels significantly increased only in fenofibrate treated rats both in skeletal muscle and in liver in both normal diet and high fat fed rats. Serum adiponectin significantly increased only in rosiglitazone treated rats in both normal diet and high fat fed groups, but elevated adiponectin did not increase AMPK activity. The results suggest that the activities of MCD and ACC are not regulated by AMPK in muscle, heart, and liver of either normal diet or high-fat fed prediabetic OLETF rats treated with PPAR-γ and -α agonists. Furthermore, our results indicate that rosiglitazone and fenofibrate modulate the MCD activity at post-transcriptional level and at transcriptional level, respectively.

**Key Words:** malonyl-CoA decarboxylase (MCD), acetyl-CoA carboxylase (ACC), AMP-activated protein kinase (AMPK), PPAR-γ and PPAR-α agonists, rosiglitazone, fenofibrate, MCD mRNA

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## I. Introduction

The role of malonyl-CoA in regulating fatty acid oxidation in rat liver[1, 2, 3], skeletal[4, 5, 6] and cardiac muscle[7, 8, 9] has been intensively investigated. Malonyl-CoA, which is produced by acetyl-CoA carboxylase (ACC) and degraded by malonyl-CoA decarboxylase (MCD), is a potent inhibitor of carnitine palmitoyltransferase-1 (CPT-1). Recent studies have demonstrated that decreases in muscle maloyl-CoA concentration have been related to activation of AMP-activated protein kinase (AMPK), which phosphorylates and inhibits acetyl-CoA carboxylase, the rate limiting enzyme in malonyl-CoA formation, resulting in a decrease in malonyl-CoA production and an increase in fatty acid oxidation rates[10, 11]. Conversely, activation

of AMPK phosphorylates and activates MCD, an enzyme that catalyzes acetyl-CoA synthesis, also resulting in a decrease in malonyl-CoA production and increase in fatty acid oxidation rates[6]. A key kinase responsible for the control of ACC and MCD activity is AMPK. Thus, AMPK is an important regulator of fatty acid oxidation in muscle and heart, since it phosphorylates and inactivates ACC and activates MCD activity, resulting in a decrease in malonyl-CoA production and an increase in fatty acid oxidation.

Rosiglitazone and fenofibrate, known as specific agonists of peroxisome proliferator activated receptor-γ (PPAR-γ) and -α (PPAR-α), improve insulin resistance in diabetic animals[12, 13, 14], and type 2 diabetes and insulin resistance syndrome patients[15, 16]. This action results from lowering blood lipids, such as triglyceride, cholesterol, and free fatty acid, which is significantly related with insulin resistance[17, 18]. In our previous study (not published) in 28 weeks old of OLETF rats, rosiglitazone and fenofibrate significantly increased MCD activities in muscles, hearts, and livers, suggesting that these two compounds lower the blood lipids at least partly through increasing MCD activity.

To our knowledge, regulations of MCD and ACC activities by AMPK have perhaps been best studied in hearts[9, 10], and exercising skeletal muscles[6, 9, 19], in which AMPK phosphorylates and inhibits ACC and phosphorylates and activates MCD. To date, no study has been performed how MCD and ACC are regulated by AMPK in non exercised muscles in diabetic animal models at prediabetic stage. Thus, it is important to define their roles in fatty acid metabolism in prediabetic period to prevent those at risk for diabetes from development of diabetes.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat was recently established as an animal model of congenital diabetes by Kawano et al[20]. Before 15 weeks of age, the strain manifests mild obesity, normoglycemia with hyperinsulinemia or insulin resistance, and dyslipidemia. These clinical and pathologic features resemble human prediabetes[20, 21]. The purpose of present study is to investigate potential

regulation of MCD and ACC by AMPK in high fat fed Otsaks Long-Evans Tokushima Fatty (OLETF) rats in prediabetic stage after treatments with rosiglitazone and fenofibrate. Therefore, malonyl-CoA decarboxylse, acetyl-CoA carboxylase, and the AMP-activated protein kinase activities in this study were assayed in muscles, hearts, and livers. In addition, MCD mRNA transcription level was assayed, and serum adiponectin was measured because it could activate the AMP-activated protein kinase[22].

## II. Materials and Methods

### 1. Materials

[1, 3$^{14}$C]malonyl-CoA purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), [$^{14}$C]sodium bicarbonate from Moravek Biochemicals (Brea, CA,USA), [$\gamma$-$^{32}$P]ATP from Amersham Biosciences (Piscataway, NJ, USA), mouse adiponectin RIA kit and rat insulin RIA kit from Linco Research, Inc. (St. Charles, MO, USA). TaqMan EZ RT-PCR Core Reagents and the primers and probes of MCD and cyclophilin purchased and biosynthesized from Applied Biosystems (Foster City, CA, USA). The AMARAASAAALARRR (AMARA) peptide was synthesized and purchased from Peptron Inc. (Taejeon, Korea). High fat rat diet purchased from BioGenomics, Inc. (Harlan, CA, USA). Whole blood glucose analyzer was obtained from Johnson & Johnson (Milpitas, CA, USA). Protease inhibitors, phosphotase inhibitors and all other reagents are purchased from Sigma Chemical (St. Louis, MO, USA).

### 2. Animals and dietary

Four weeks of aged male Long-Evans Tokushima Otruka (LETO) rats (n=20) and Otruka Long-Evans Tokushima Fatty (OLETF) rats (n= 60) donated from Otuska Pharmaceuticals (Japan) and were group-housed until experiment. At 14 weeks of age, the rats were randomly assigned to 1 of 7 dietary regimens: 1) LETO (n=10) standard rat diet, 2) OLETF (n=10) standard rat diet, 3) OLETF (n=10) standard rat diet with rosiglitazone (4 mg/kg/d), 4) OLETF (n=10) standard rat diet with

fenofibrate (100 mg/kg/d), 5) OLETF (n=10) high-fat diet, 6) OLETF (n=10) high-fat diet with rosiglitazone (4 mg/kg/d), 7) OLETF (n=10) high-fat diet with fenofibrate (100 mg/kg/d). The formula of standard diet is approximately 3.6 kcal/g, and the relative amounts are 21% from protein, 12.5% from fat, 66.5% from carbohydrate; and the formula of high fat diet is approximately 5.0 kcal/g, and the relative amounts are 21% from protein, 66.5% from fat, 12.5% from carbohydrate. All rats were kept in the Department of Animal Experiment of Clinic Medical Research Center, Yonsei University Medical College, under controlled conditions of $23 \pm 1^0$C and 12:12-h light-dark cycle, and allowed to free access to respective diets and water ad libitum. All rats were cared for the period of entire experiments by the Guidelines of Animal Experiments recommended by Korean Academy Sciences.

### 3. Food intake, body weight, and oral glucose tolerance test

Food intake was checked every day, and body-weight checked every 3-day period throughout experimental period. At the beginning and the end of experiment, oral glucose tolerance tests (OGTT) were performed to confirm that all rats had normal glucose tolerance. Rats were orally given glucose (2 g/kg) and the blood samples were collected from the tails at 0, 30, 60, 90, 120 min after glucose load. The glucose levels were measured with glucose analyzer (Milpitas, CA, USA).

### 4. Tissue sampling and measurement of fat mass

After two weeks of high fat feeding and treatments with rosiglitazone and fenofibrate, the animals were anesthetized with ether and sacrificed for tissue sampling. Blood was collected by cardiac puncture for triglyceride, total cholesterol, free fatty acid, and adiponectin assays. Abdominal subcutaneous fat and epididymal fat pads were surgically removed after mid-abdominal incision was done. The weight of each dissected fat mass was weighed immediately. Skeletal muscles, hearts, livers, and pancreas were isolated and immediately freeze-clamped in liquid nitrogen, and stored at $-80^0$C until assays.

## 5. Serum lipid and adiponectin concentraions

The levels of triglyceride, total cholesterol, free fatty acid were measured using infinity triglycerides reagent, infinity cholesterol reagent, and ACS-ACOD enzyme method (NEFA ZYME-S, Aiken, Japan). Serum adiponectin concentration was assayed using mouse adiponectin RIA kit according to the manual's instruction.

## 6. Extraction of AMPK and ACC

Approximately 300 mg of frozen tissue were homogenized with a buffer containing Tris.HCl (10 mM), mannitol (200 mM), NaF (50 mM), EDTA (1 mM), 2-mercaptoethanol (10 mM), pH 7.5. Aprotinin, leupetptin, and antitrypsin, three preoteolytic enzyme inhibitors, were added at a concentration of 5 mg/L. The homogenate was immediately centrifuged at 48,000 g for 30 min at $4^0$C. The ACC and AMPK were precipitated from the supernatant by addition of 144 mg ammonium sulfate/ml and by stirring for 60 min on ice. The precipitate was collected by centrifugation at 48,000 g for 30 min. The pellet was dissolved in 10% of the original volume of the homogenate buffer and was centrifuged again to remove insoluble protein. The supernatant was used for determination of ACC and AMPK activities.

## 7. ACC assay

ACC activity was determined by measurement of the rate of incorporation of [$^{14}$C]bicarbonate into acid-stable compounds (malonyl-CoA). Final concentrations of assay components were 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2.5 mM $MnCl_2$, 2 mM dithiothreitol, 0.125 mM acetyl-CoA, 4 mM ATP, 12.5 mM $NaHCO_3$, 2 μCi of [$^{14}$C]$NaHCO_3$, and 1 mg/ml fatty acid free bovine serum albumin, pH 7.5. The reactions were started by addition of 20 μl of the enzyme preparation. Final volume of the assay mixture was 200 μl. After a 10-min incubation at $37^0$C, the reaction was stopped by addition of 50 μl of 5 M HCl. After centrifugation, 150 μl were transferred to a scintillation vial and evaporated to dryness at $80^0$C. The residue was dissolved in 0.5 ml water and then mixed with 5.5 ml scintillation solution for determination of radioactivity. ACC activity was

expressed as the amount of malonyl-CoA produced per minute per milligram protein.

## 8. AMPK assay

AMPK activity was measured by following the incorporation of $^{32}P$ into a synthetic peptide. Briefly, the reaction mixture composed of HEPES-NaOH (40 mM), NaCl (80 mM), glycerol (8%, wt/vol), EDTA (0.8 mM), dithiothreitol (0.8 mM), $MgCl_2$ (5 mM), AMARAASAAA-LARRR (AMARA) peptide (0.2 mM), ATP (0.2 mM), [$\gamma$-$^{32}P$]ATP (2 μCi), AMP (0.2 mM). The reactions were started by addition of 10 μg of the enzyme preparation. Final volume of the assay mixture was 25 μl. This mixture was incubated for 5 min at $37^0C$. From this incubation mixture, 15 ul were spotted on 1-$cm^2$ phosphocellulose paper. The paper was then washed four times for 10 min each with 150 mM phosphoric acid, followed by a 5-min acetone wash. Papers were then dried and counted for radioactivity. AMPK activity was expressed as nmole $^{32}P$ incorporated in the AMARA peptide per minute per milligram protein.

## 9. MCD activity assay

Frozen muscles (300 mg) are powdered in liquid nitrogen, weighed, and then homogenized in a glass homogenizer in 30 volumes of a buffer composed of 0.1 M Tris-HCl (pH 8.0), 2 mM PMSF, 5 μM aprotinin, 5 μM leupeptin, and 5 μM pepstatin A, with the addition of 40 mM β-glycerophosphate, 40 mM NaF, 4 mM NaPPi, and 1 mM $Na_3VO_4$ to inhibit phosphatase activity unless otherwise indicated. The homogenized muscles are then centrifuged at 500 × g (2,500rpm) for 10 min. To the supernatant, powdered $(NH_4)_2SO_4$ is slowly added with stirring until 40% (243 gm/L of Ammonium Sulfate) saturation is achieved. The mixture is stirred for 1 hour on ice and centrifuged at 14,000 × g for 10 min. The supernatant from this spin is treated with additional $(NH_4)_2SO_4$ until 55% (351 gm/L of Ammonium Sulfate) saturation is achieved. The mixture is recentrifuged at 14,000 × g (13,000rpm). The resultant pellet fraction is dissolved in 0.1 M Tris-HCl (pH 8.0) and stored at $4^0C$ for use in all further studies. The enzyme activity was assayed by measuring the amount of $^{14}CO_2$ generated from [3-$^{14}C$]malonyl-CoA. The reaction mixture contains

10umol of Tris-HCl buffer (pH 8.0), 0.01umol of DTE, 0.02 umol of [3-$^{14}$C]malonyl-CoA. Reaction mixture and enzyme in a total volume of 0.1ml were incubated for 10 min at 37$^{0}$C. The $^{14}CO_2$ generated were trapped in 2N KOH and assayed by liquid scintillation spectrometry using a counting fluid consisting of 30% ethanol in toluence containing 4 g of Omnifluor per liter. The enzyme activity was calculated as nmole per mg protein per minute.

## 10. Real-time RT-PCR

RNA was extracted using TRIzol Reagent (GIBCO BRL) according to the manual's instruction. Specific quantitative assays for MCD and cyclophilin were designed from the rat sequences available in GenBank. Primers and probes (shown in table 1) were designed around specific splice junctions, preventing the recognition of any contaminating genomic DNA. The level of transcripts for the constitutive housekeeping gene products cyclophilin was quantitatively measured in each sample to normal control (LETO rat) for sample-to-sample differences in RNA concentration. Reaction for each sample (RNA) was triplicated, and the reagent mix was prepared according to the manual's instruction. Thermal cycling parameters are shown in table 2. MCD mRNA expression is calculated by comparative CT (threshold cycle) method and reported as ratio of MCD transcripts per cyclophilin transcript molecules.

**Table 1.** Primer and probe sequences used for real time RT-PCR

| Gene | Primer/Probe | Sequence |
|---|---|---|
| MCD | Forward | 5'-CGGCACCTTCCTCATAAAGC-3' |
| | Reverse | 5'-GGGTATAGGTGACAGGCTGGA-3' |
| | Probe | 5'-FAM-AGTGGTCAAGGAGCTGCAGAAGGAGTTT-TAMRA-3' |
| Cyclophilin | Forward | 5'-CTGATGGCGAGCCCTTG-3' |
| | Reverse | 5'-TCTGCTGTCTTTGGAACTTTGTC-3' |
| | Probe | 5'-FAM-CGCGTCTGCTTCGAGCTGTTTGCA-TAMRA-3' |

**Table 2.** Thermal cycling parameters

| Thermal Cycler | Times and Temperatures | | | | |
|---|---|---|---|---|---|
| | Initial Step | RT | Deactivation | Each of 40 cycles | |
| | | | | Melt | Anneal/Extend |
| ABI Prism 7700 Sequence Detection System | HOLD | HOLD | HOLD | CYCLE | |
| | 2 min 50°C | 30 min 60°C | 5 min 95°C | 20 seconds 94°C | 1 min 62°C |

## 11. Statistical analysis

Data are presented as means ± SD or means ± SEM. Statistically significant differences between groups calculated by Tukey's Multiple Comparison Test of One-Way ANOVA. A value of $P < 0.05$ is considered significant.

# III. Results

## 1. Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT, 2 g/kg body weight) was performed at the beginning and the end of the experiment, at the rat age of 14 weeks and 16 weeks after 2 weeks of treatment. All rats had normal glucose tolerance (Fig. 1A, B).

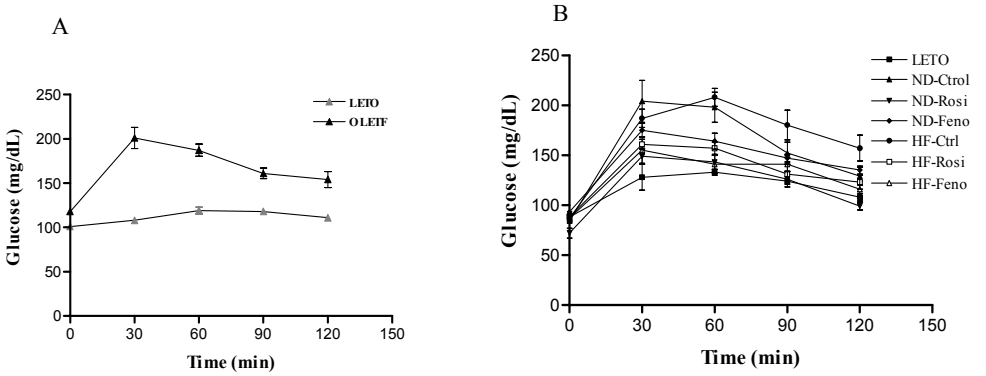


**Fig. 1.** Results of oral glucose tolerance test (OGTT, 2 g/kg body weight) performed at the beginning and the end of experiment. Fig. A shows the result of OGTT

performed at the beginning at the rat age of 14 weeks, and Fig. B shows the result of OGTT at the end of the rat age of 16 weeks after 2-week treatment of PPAR-γ and -α agonists. All rats had normal glucose tolerance. Results are mean ± SD of 6~10 rats in each group. Normal diet (ND), high fat diet (HF). Control (Ctrl), Rosiglitazone (Rosi), Fenofibrate (Feno), Long-Evans Tokushima Otruka (LETO).

## 2. Food intake and body weight changes

Food-intake was dramatically decreased in fenofibrate treated rats in both normal diet and high fat fed rat (Fig. 2A). When the food-intake is calculated by calorie, high fat (5.0 kcal/g) fed rats took significantly much more calories than normal diet (3.08 kcal/g) fed rats. In terms of calories, 52% (49.3 kcal), 73% (58 kcal), and 57% (42.6 kcal) more calories each rat per day were consumed in high fat fed control, rosiglitazone, and fenofibrate treated rats compared to those in corresponsive normal diet fed animals (Fig. 2B).

Body weight in high fat fed control and rosiglitazone treated rats significantly increased compared to fenofibrate treated rats in both normal diet and high fat fed animals ($P < 0.05$, Fig. 2C). High calorie intake had significant impact on body weight gains in high fat fed control and rosiglitazone treated rats compared to corresponsive normal diet fed rats, respectively.

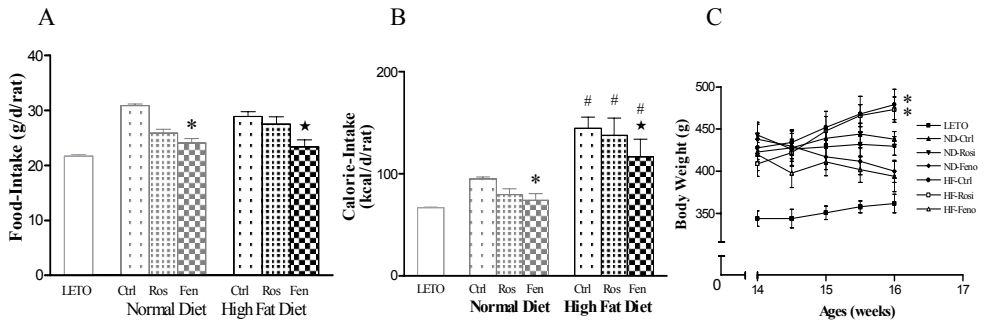


**Fig. 2.** Food and calorie intakes and body weight changes. A shows the food-intake each rat per day, *, $P < 0.01$, significantly different from control. B shows the calorie intake each rat per day, *, $P < 0.05$ from control and rosigitazone treated groups in normal diet fed rats; *, $P < 0.01$, significantly different from controls in both normal

diet and high fat fed rats, respectively. #, P < 0.01, significantly from corresponsive control, rosiglitazone, and fenofibrate treated groups in normal diet fed rats. C shows the body weight changes with the time courses. Body weights in control and rosiglitazone treated rats in high fat fed groups significantly increased compared to fenofibrate treated rats in both normal and high fat diet fed groups. *, P < 0.01 vs. fenofibrate treated groups in both normal diet and high fat diet fed animals. Results are means ± SD of 6~10 rats in each group.

### 3. Fat mass and body weight

In normal diet fed OLETF group, subcutaneous and epididymal fat masses did not show significant changes. In high fat fed group, significant difference was found in both control and rosiglitazone treated rats compared to fenofibrate treated animals. High calorie had dramatic effects on subcutaneous and epididymal fat in high fat fed control and rosiglitazone treated rats compared to corresponsive normal diet fed animals. Nearly *2-fold* and *2.3-fold* increases in subcutaneous fat were found in high fat fed control and rosiglitazone treated rats compared to corresponsive normal diet fed rats. Epididymal fat increased 75% and 78% in high fat fed control and rosiglitazone treated rats compared to corresponsive normal diet fed rats (Table 3).

**Table 3.** Fat Mass and Body Weight after two weeks of treatment (gram)

| | | Normal Diet | | | High Fat Diet | | |
|---|---|---|---|---|---|---|---|
| | LETO | Control | Rosiglitazone | Fenofibrate | Control | Rosiglitazone | Fenofibrate |
| SQ. Fat | 4.8 ± 0.3 | 8.6 ± 0.6 | 7.6 ± 0.5 | 7.3 ± 0.7 | 15.9 ± 0.6 * | 17.5 ± 1.4 * | 8.4 ± 0.8 ★ |
| Epid. Fat | 4.1 ± 0.1 | 5.7 ± 0.5 | 5.4 ± 0.3 | 5.3 ± 0.3 | 10.0 ± 0.3 * | 9.6 ± 0.9 * | 5.4 ± 0.4 ★ |
| B. Wt. | 362 ± 5 | 438 ± 10 | 430 ± 7 | 400 ± 15 | 479 ± 5 | 473 ± 13 | 394 ± 6 |

Values are means ± SEM; N = 6~10 rats. Blood samples were taken from animals at the time of death. Both subcutaneous (SQ) and epididymal (Epid) fat masses were significantly increased in control and rosiglitazone treated groups in high fat diet fed

animals compared with corresponsive normal diet fed rats and fenofibrate treated rats in high fat diet fed animals. *, P < 0.01, significantly different from corresponsive normal diet fed rats. ★, P < 0.01 vs. control and rosiglitazone treated groups in high fat diet fed rats.

Percent of the sum of subcutaneous and epididymal fat masses to the body weight showed that increases in fat masses in high fat fed control and rosiglitazone treated rats were most significant compared to all other experiments animals (Fig. 3).

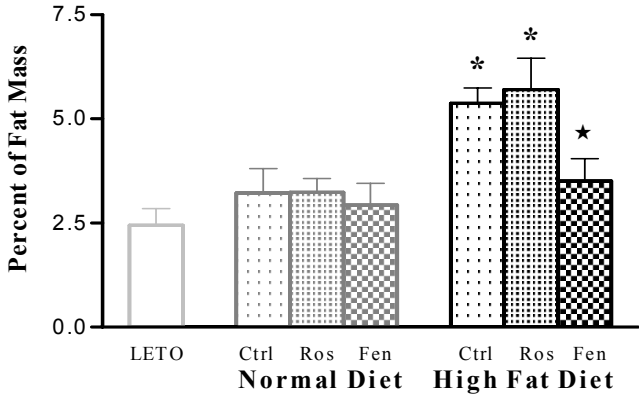


**Fig. 3.** Percent of sum of subcutaneous and epididymal fat pads to body weight. *, P < 0.01 vs. corresponsive control and rosiglitazone treated groups in normal diet fed rats. ★, P < 0.01 vs. control and rosiglitazone groups in high fat fed rats

## 4. Serum triglyceride, free fatty acid, and cholesterol

Triglyceride greatly increased in normal diet fed control rats (93 ± 15), but decreased 54% and 45% by treatments with rosiglitazone (43 ± 5) and fenofibrate (51 ± 4), respectively. Likewise, triglyceride in high fat fed control rats (133 ± 24) dramatically increased, decreased again 56% and 44% by treatments with rosiglitazone (59 ± 4) and fenofibrate (76 ± 9), respectively (Fig. 4A). High calorie had significant effect on triglyceride concentration in normal diet and high fat diet control groups, but no significant effects in both rosiglitazone and fenofibrate treated rats. In normal diet fed group, free fatty acid dramatically increased in control rats

(1880 ± 164), and decreased 62% and 33% by treatment with rosiglitazone (708 ± 95) and fenofibrate (1258 ± 106), respectively. In high fat fed group, free fatty acid also greatly increased in control rats (2710 ± 318), again decreased 55% and 40% by treatment with rosiglitazone (1224 ± 160) and fenofibrate (1638 ± 276), respectively. High calorie had significant effects on free fatty acid concentration in OLETF rats. Free fatty acid increased 43%, 37%, and 49% in high fat fed control, rosiglitazone, and fenofibrate treated groups compared to corresponsive normal diet fed control, rosiglitazone, and fenofibrate treated groups, respectively (Fig. 4B). No significant difference in cholesterol was found in normal diet fed group. In high fat fed group, cholesterol concentration greatly increased in control rats (124 ± 6), and decreased 27% by treatment with fenofibrate (91 ± 3) (Fig. 4C).

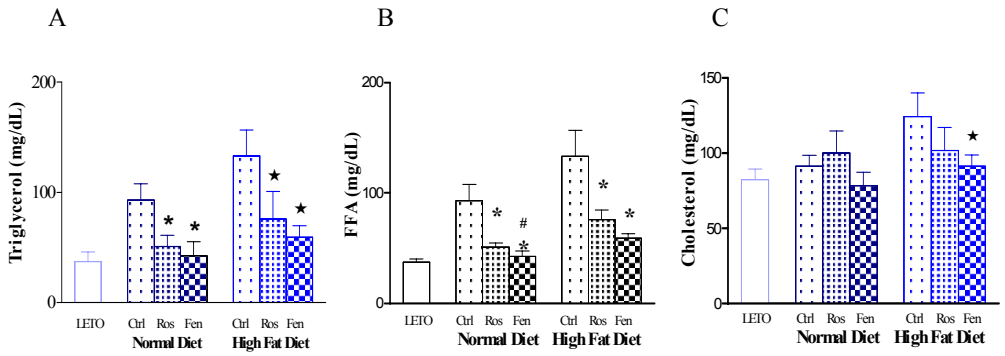


**Fig. 4.** Measurement of serum triglyceride, free fatty acid (FFA), total cholesterol. Blood samples were taken from hearts at the time of death. In the left figure, * and ★ $P < 0.01$, significantly different from corresponsive control groups in both normal diet and high fat fed animals. In the middle figure, *, $P < 0.01$, significantly different from corresponsive control groups in both normal diet and high fat fed animals; # $P < 0.01$ vs. rosiglitazone treated rats in normal diet fed rats. In the right figure, *, $P < 0.01$, significantly different from control group in high fat fed rats. Values present means ± SEM of 6-10 rats in each group.

## 5. AMPK, MCD, and ACC activities in skeletal muscle

Rosiglitazone and fenofibrate treatments had no effects on AMPK activities either

in normal diet or in high fat diet fed OLETF rats (Fig. 5A). MCD activities increased *2.4-fold* in rosiglitazone (3.34 ± 0.43) and *1.8-fold* in fenofibrate (2.54 ± 0.21) treated rats compared to control (1.38 ± 0.14) in normal diet fed rats, and *2-fold* in rosiglitazone (2.40 ± 0.24) and *4.5-fold* in fenofibrate (5.25 ± 0.88) treated rats compared to control (1.18 ± 0.28) in high fat fed groups (Fig. 5B). ACC activities did not significantly changed (Fig. 5C).

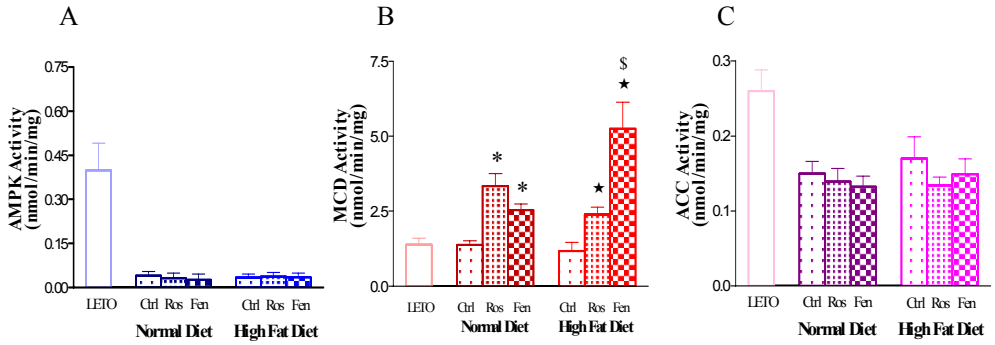


**Fig. 5.** AMP-activated protein kinase (AMPK), malonyl-CoA decarboxylase (MCD), and acetyl-CoA carboxylase (ACC) activities in skeletal muscle. AMPK activities did not show significant changes in all experimental OLETF rats. MCD activities significantly increased in both normal diet and high fat fed groups compared to corresponsive controls. *, $P < 0.001$ vs. corresponsive controls; $, $P < 0.001$ vs. control and rosiglitazone treated rats in high fat fed group. ACC activities did not show significant changes in all experimental OLETF rats. Values are means ± SEM (N = 6-10).

## 6. AMPK, MCD, and ACC activities in heart

AMPK activity increased nearly *2.3 fold* in fenofibrate (2.22 ± 0.26) treated group compared to both control (0.97 ± 0.15) and rosiglitazone (0.98 ± 0.22) treated groups in normal diet fed rats. Likewise, AMPK activity in fenofibrate treated group increased 68% and 37% compared to control (2.28 ± 0.16) and rosiglitazone (2.78 ± 0.47) treated groups in high fat fed rats, respectively. High calorie had significant effects on AMPK activity. There are remarkable increases in AMPK activity in high

fat diet fed rats compared to normal diet fed rats, *2.3-fold* increase in control, *2.8-fold* in rosiglitazone, and *1.7-fold* in fenofibrate treated groups, respectively (Fig. 6A). MCD activity in normal diet fed rats increased 28% in fenofibrate (18.50 ± 0.86) treated group compared to both control (14.50± 0.85) and rosiglitazone (14.37 ± 0.99) treated groups; In high fat fed rats, MCD activity decreased 41% in both rosiglitazone (11.91 ± 0.94) and fenofibrate (11.88 ± 0.80) treated groups compared to control (20.21 ± 1.36) (Fig. 6B). No significant differences in ACC activities in all experimental rats were found (Fig. 6C).

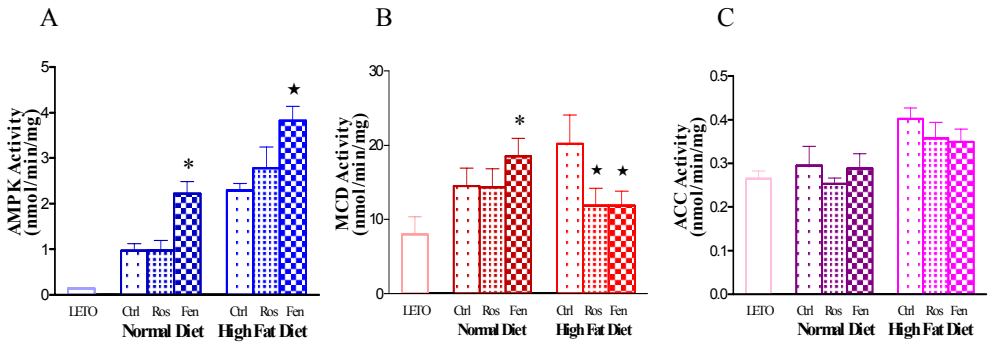


**Figure 6.** AMP-activated protein kinase (AMPK), malonyl-CoA decarboxylase (MCD), and acetyl-CoA carboxylase (ACC) activities in heart. AMPK activities in fenofibrate treated rats significantly increased in both normal diet and high fat fed rats. The enzyme activities in high fat fed groups significantly increased compared to those of corresponsive control, rosiglitazone, and fenofibrate treated rats in normal diet fed groups. MCD activities dramatically increased in fenofibrate treated rats compared to control and rosiglitazone treated rats in normal diet fed groups, but significantly decreased in rosiglitazone and fenofibrate treated rats in high fat fed groups. *, $P < 0.01$ vs. control and rosiglitazone treated rats in normal diet fed rats, and $< 0.001$ vs. control in high fat fed groups. ACC activities did not show significant changes in all experimental animals.

## 7. AMPK, MCD, and ACC activities in liver

AMPK activity in fenofibrate (0.11 ± 0.02) treated rats decreased 72% and 66%

compared to control (0.38 ± 0.10) and rosiglitazone (0.32 ± 0.06) treated rats in high fat fed groups. High calorie had dramatic impacts on AMPK activities when treated with fenofibrate, 64% decrease in AMPK activity was found in high fat fed rats compared to normal diet fed and fenofibrate (0.30 ± 0.07) treated rats (Fig. 7A). MCD activity in fenofibrate treated rats increased *2.7-fold* (10.14 ± 0.80) and *2.5-fold* (10.14 ± 0.80) compared to control (3.80 ± 0.24) and rosiglitazone (4.12 ± 0.27) treated groups in normal diet fed rats, respectively. In high fat fed rats, *1.6-fold* increases in rosiglitazone (6.59 ± 0.27) and *2.0-fold* in fenofibrate (8.56 ± 0.28) treated rats in MCD activities were found compared to control rats (4.22 ± 0.33). High calorie increased MCD activity by 60% in rosiglitazone treated rats but no significant differences were found in both control and fenofibrate treated animals (Fig. 7B). No significant changes in ACC activities were found in normal diet fed rats. In high fat diet fed rats, ACC activity decreased 38% in rosiglitazone (0.21 ± 0.03) and 87% in fenofibrate (0.04 ± 0.01) treated rats compared to control rats (0.34 ± 0.03). High calorie increased ACC activity by 54% in control groups (normal vs. high fat: 0.22 ± 0.01 vs. 0.34 ± 0.03) and decreased enzyme activity by *5.5-fold* (normal vs. high fat: 0.22 ± 0.04 vs. 0.04 ± 0.01) in by treatment with fenofibrate (Fig. 7C).

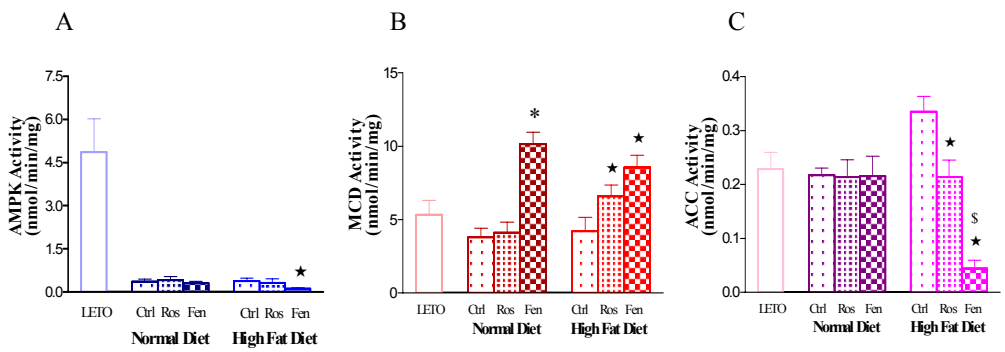


**Fig. 7**. AMP-activated protein kinase (AMPK), malonyl-CoA decarboxylase (MCD), and acetyl-CoA carboxylase (ACC) activities in liver. AMPK activity decreased only in fenofibrate treated rats in high fat fed groups. *, P < 0.01 vs. control and rosiglitazone treated rats in high fat diet fed groups. MCD activities significantly

increased in fenofibrate treated rats in normal diet fed groups and both rosiglitazone and fenofibrate treated rats in high fat fed groups. *, $P < 0.001$ vs. control and rosiglitazone treated rats in normal diet fed groups, and < 0.01 vs. control in high fat fed groups. ACC activities significantly decreased in both rosiglitazone and fenofribrate treated rats compared to control in high fat fed groups. The enzyme activity in fenofibrate treated rats further decreased compared to rosiglitazone treated rats. *, $P < 0.001$ vs. control; $, $P < 0.001$ vs. rosiglitazone treated rats. Results are means ± SEM of 6-10 animals.

## 8. MCD mRNA expression in skeletal muscle and liver

MCD mRNA expression increased *2.6-fold* in fenofibrate (0.73 ± 0.08) treated rats compared to both control (0.28 ± 0.03) and rosiglitazone (0.31 ± 0.04) treated rats in normal diet fed animals. Likewise, MCD mRNA expression increased 27% in fenofibrate (0.80 ± 0.09) treated rats compared to both control (0.63 ± 0.04) and rosiglitazone (0.63 ± 0.05) treated rats in high fat fed animals. High calorie had significant effects on MCD mRNA expression in control and rosiglitazone treated groups, but no significant effects on fenofibrate treated groups. MCD mRNA expression increased *2.3-fold* in controls (normal vs. high fat: 0.28 ± 0.03 vs. 0.63 ± 0.04) and *2.0-fold* in rosiglitazone (normal vs. high fat: 0.31 ± 0.04 vs. 0.63 ± 0.05) treates groups in high fat fed rats compared to normal diet fed rats (Fig. 8A).

In liver tissues, MCD mRNA expression levels significantly increased in fenofibrate treated rats in both normal diet and high fat diet treated animals. MCD mRNA increased *3.1-fold* (2.21 ± 0.23) in normal diet and *2.5-fold* (1.47 ± 0.07) in high fat fed rats compared to normal diet (0.71 ± 0.08) and high fat diet (1.47 ± 0.07) controls, respectively. Unexpectedly, 34% decreases in MCD mRNA expression were observed in high fat fed rats compared to normal diet fed rats when treated with fenofibrate (Fig. 8B).

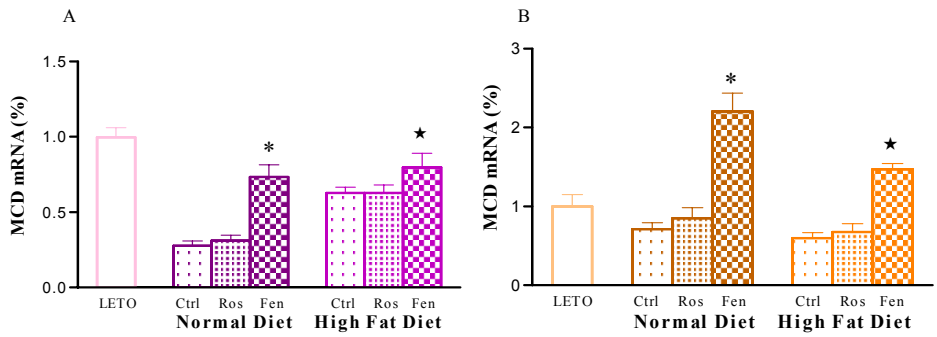


**Fig. 8.** MCD mRNA expression levels in skeletal muscles and livers in normal diet and high fat fed OLETF rats compared to LETO (normal control) rats. In muscles, MCD mRNA expression levels significantly increased only in fenofibrate treated groups in both normal diet and high fat fed groups. * and ★, $P < 0.01$ vs. corresponsive control and rosiglitazone treated rats. Likewise, in livers MCD mRNA expression levels significantly increased only in fenofibrate treated groups in both normal diet and high fat fed groups. * and ★, $P < 0.01$ vs. corresponsive control and rosiglitazone treated rats.

## 9. Serum adiponectin concentration

Serum adiponectin concentration increased only in rosiglitazone treated rats, 57% (6.36 ± 0.40) in normal diet and 59% (9.82 ± 0.38) in high fat fed rats compared to respective controls (normal: 4.06 ± 0.26; high fat: 6.18 ± 0.24). High calorie had significant effects on control and rosiglitazone treated rats. In control groups, adiponectin increased 52% in high fat fed rat compared to normal diet fed rats; in rosiglitazone treated groups, adiponectin increased 54% in high fat fed rats compared to normal diet fed rats (Fig. 9).

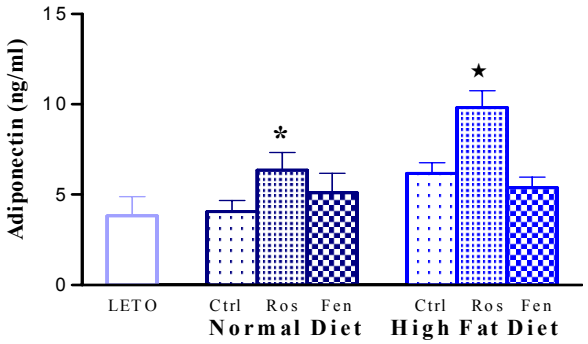


**Fig. 9**. Serum adiponectin concentration significantly increased only in rosiglitazone treated rats in both normal diet and high fat fed groups. * and ★, $P < 0.01$ vs. corresponsive control and fenofibrate treated rats. Results are means ± SEM of 6 ~ 8 animals in each group.

## IV. Discussion

The major findings of this study are as follows. **1)** In skeletal muscles, AMPK activities dramatically decreased in OLETF rats compared to normal control LETO rats. No difference in AMPK activities between normal diet and high fat fed animals in OLETF rats was found. MCD activities significantly increased in rosiglitazone and fenofibrate treated OLETF rats in both normal diet and high fat fed animals compared to normal control LETO rats. Enzyme activity in fenofibrate treated rats was significantly higher in high fat fed animals than in normal diet fed rats. ACC activities in OLETF rats decreased in OLETF rats compared to normal control LETO rats. But no difference in ACC activities between normal diet and high fat fed animals in OLETF rats was found. **2)** In hearts, AMPK activities dramatically increased in all OLETF rats compared to normal control LETO rats. AMPK activities were significantly higher in fenofibrate treated rats than in both control and rosiglitazone treated rats either in normal diet or in high fat fed animals. In addition, enzyme activities were much higher in high fat fed control, rosiglitazone , and fenofibrate treated rats than in corresponsive normal diet fed animals. MCD activities significantly increased in all OLETF rats compared to normal control

LETO rats. MCD activity significantly increased in fenofibrate treated rats compared to control and rosiglitazone treated animals in normal diet fed rats. But the enzyme activity significantly increased in control group compared to the other two groups in high fat fed animals. ACC activities showed no significant alterations in LETO and all OLETF rats. **3)** In livers, AMPK activities dramatically decreased in all OLETF rats compared to normal control LETO rats. In normal diet fed animals, no significant changes in AMPK activities were found. In high fat fed animals, enzyme activity dramatically decreased in fenofibrate treated rats compared to control and rosiglitazone treated rats. In addition, AMPK activity in fenofibrate treated rats was much lower in high fat fed rats than in normal diet fed animals. MCD activities decreased in normal diet fed OLETF control and rosiglitazone treated groups, and in high fat fed control group, but dramatically increased in fenofibrate treated rats in normal diet fed group and rosiglitazone and fenofibrate treated rats in high fat fed groups. Enzyme activity in rosiglitazone treated groups was much higher in high fat fed rats than in normal diet fed animals. ACC activity in high fat fed control group significantly increased compared to normal control LETO rats, normal diet fed OLETF control and high fat fed rosiglitazone group. Enzyme activity in high fat fed fenofibrate treated rats dramatically decreased compared to normal control LETO rats, rosiglitazone treated animals, and normal diet fed fenofibrate treated rats. **4)** In skeletal muscles, MCD mRNA transcripts decreased in all OLETF rats compared to normal control LETO rats. MCD mRNA levels significantly increased in fenofibrate treated rats compared to control and rosiglitazone treated groups either in normal diet fed rats or in high fat fed animals. MCD mRNA levels significantly increased in high fat fed control and rosiglitazone treated groups compared to corresponsive normal diet fed animals, but no dramatic difference in fenofibrate treated rats was found between normal diet and high fat fed animals. **5)** In livers, MCD mRNA levels significantly increased in fenofibrate treated OLETF rats in both diet groups. In addition, mRNA transcription was much higher in normal diet fed rats than in high fat fed animals.

The AMPK activity was liver > skeletal muscle > heart in LETO rats, and heart >

liver > skeletal muscle in both normal diet and high fat fed OLETF rats. MCD activity was heart > liver > skeletal muscle in both LETO rats and all OLETF rats. ACC activity skeletal muscle = heart > liver in LETO rats, and heart > liver > skeletal muscle except high fat fed fenofibrate treated rats in which ACC activity was heart > skeletal muscle > liver.

The results of present study indicate that the increases in MCD activities paralleled with the increases in AMPK activities, and no significant changes in ACC activities were observed in hearts. As a result, the concentration of malonyl-CoA probably decreases in heart, leading to increases in fatty acid oxidation in myocardium. It is consistent with the previous studies in which the decrease in concentration of malonyl-CoA resulted from the activation of AMPK, which phosphorylates MCD and ACC, leading to accelerated degradation of malonyl-CoA and reduction of malonyl-CoA synthesis[6, 10, 23].

In skeletal muscles, ACC and AMPK activities are significantly lower in all OLETF rats than in LETO rats. No significant differences in ACC and AMPK activities between normal diet and high fat fed OLETF rats were found. MCD activities in rosiglitazone and fenofibrate treated OLETF rats dramatically increased compared to those of LETO and OLETF control groups. This result indicates that the increases in MCD activities did not accompanied with the increases in AMPK activities in skeletal muscles. This result was controversy to the previous studies in which MCD is an substrate of AMPK and regulated by AMPK[6, 10, 23], but consistent with the study by Habinowski SA, et al. in which activation of AMPK in skeletal muscles had no any effect on MCD activity and did not phosphorylate in vitro either recombinant MCD or MCD immunoprecipitated from skeletal muscle and heart[24].

A novel finding in this study was that in liver tissues, increase in MCD activity in fenofibrate treated rats did not accompanied with the increase in AMPK activity in normal diet fed groups. Surprisingly, MCD activity in fenofibrate treated rats still increased even AMPK activity dramatically decreased in high fat fed groups. More

surprisingly, decrease in AMPK activity accompanied with decrease in ACC activity in high fat fed animals. Earlier studies by Haejoe Park[23] and Carlson and Winder[25] et al reported that increases in MCD and AMPK activities and decreases in ACC activity were observed in rat livers after exercises. This difference between our result and those by earlier studies could be due to the difference of sedentary and exercise. But it is difficult to understand what causes the parallel decreases in AMPK and ACC activities at the same time. The reason for this result is unclear, but it could reflect the fact that the condition in livers of high fat fed rats is much more complicated that that of normal diet fed animals. The other reason is that there possibly exists unknown factors that regulate ACC activity in certain circumstances as it is in an early study by Neil B et al[5] in which they reported that ACC is regulated both allosterically by cytosolic citrate and covalently by AMPK phosphorylation in skeletal muscles. In high fat fed condition, there are more factors involved in lipid metabolism, leading to changes in the expression of genes encoding other enzymes of lipid partitioning and other proteins.

To investigate the exact mechanism how ACC and MCD regulated by AMPK, further study in which comparison of phosphorylated ACC, MCD, and AMPK should be examined is necessary. In skeletal muscles, the effect on MCD mRNA transpcrition between rosiglitazone and fenofibrate is substantially different. Rosiglitazone had almost no effect on MCD mRNA transpcrition, suggesting that elevated MCD activities in skeletal muscles both normal diet and high fat fed rats were regulated at post transcription levels. But fenofibrate affected MCD activity begun from transcription level, the MCD mRNA concentration substantially increased in fenofibrate treated animals. This effect is much more striking in liver tissues. In liver tissues, again rosiglitazone had no significant effect on MCD mRNA transpcrition, increase in MCD activity accompanied with increase in MCD mRNA concentration in fenofibrate treated animals both normal diet and high fat fed rats.

Serum adiponectin concentration significantly increased in rosiglitazone treated groups both normal diet and high fat fed rats. It is consistent with the studies by Tsao,

T. S.[26], and Yu, J. G.[27] et al in which they reported that thiazolidinediones stimulates adiponectin production by adipocytes and increase its concentration in plasma. But elevated concentration of adiponectin did not increase AMPK activity and decrease ACC activity in skeletal muscles, hearts, and livers in our study. It is controversy to the studies by Eva Tomas[28] and T. Yamauchi[22] et al in which they reported that adiponectin increase AMPK activity and ACC phosphorylation in skeletal muscles and livers. This controversy between our study and others remains to be determined.

## V. Conclusion

According to the results observed in the study, we can conclude that body weight increased significantly in high fat fed control and rosiglitazone treated rats compared to all the other OLETF animals. This body weight gain was mainly due to the excess calorie intake which in turn substantially increased subcutaneous and epididymal fat masses. Lipid-lowering effects were remarkable in triglyceride and free fatty acid both rosiglitazone and fenofibrate treatments, but the greatest impact was observed in fenofibrate treated rats. Adiponectin concentration significantly increased in rosiglitazone treated rats, but this elevated adiponectin did not seem to stimulate AMPK activities in three different tissues we examined as it did in other studies.

Increase in MCD activity accompanied with increase in AMPK activity in hearts, but the same result had not been observed in skeletal muscles in which MCD activity dramatically increased in rosiglitazoen and fenofibrate treated rats, but AMPK activities remained unchanged. In contrast, the opposite effect in enzyme activities between MCD and AMPK was observed in liver tissues in which in normal diet fed rats, MCD activities increased in fenofibrate treated animals, but no increased AMPK activity was found; in high fat fed rats, the increased MCD activity in rosiglitazone treated rats was not parallel with the increased activity of AMPK which remained unchanged. Even increased MCD activity in fenofibrate treated animals accompanied with decreased AMPK activity in high fat fed livers.

Rosiglitazone had little effects on MCD mRNA transcription both in skeletal muscle and liver. High fat had significant effects on MCD mRNA transcription in skeletal muscle but not in liver except fenofibrate treated rats. Unlike rosiglitazone, fenofibrate had dramatic effects on MCD mRNA transcription both normal and high fat diet fed animals in which it increased MCD mRNA level by *2.4-fold* and *3.1-fold* compared to normal diet fed control rats in skeletal muscle and liver, respectively, and 27% and *2.5-fold* compared to high fat fed control rats in skeletal muscle and liver, respectively.

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## Abstract (국문요약)

# OLETF 쥐에서 PPAR-γ 와 –α agonists가 MCD, ACC, AMPK 효소들의 조절에 미치는 영향

지도교수 차 봉 수

연세대학교 대학원 의과학과

조 정 산

운동을 시킨 쥐의 골격근, 심장, 간 등 조직에서 malonyl-CoA decarboxylase (MCD) 와 acetyl-CoA carboxylase (ACC) 두 효소가 AMP-activated protein kinase (AMPK)에 의해 조절이 된다는 사실은 잘 알려져 있다. PPAR-γ 와 –α agonists 가 MCD, ACC, AMPK 등 효소들의 활성도 및 이들 효소들의 조절에 대해 알아 보기 위하여, 제2형 당뇨병 모델인 OLETF 쥐의 당뇨병 전 단계에서 정상 식이 와 고지방 식이를 섭취 시키고 PPAR-γ 와 –α agonist 인 rosiglitazone (4 mg/kg/d) 과 fenofibrate (100 mg/kg/d)를 2주 간 투여 한 후 MCD, ACC, AMPK 활성도 및 MCD mRNA 발현 정도를 측정하였다. 정상 식이를 섭취시킨 쥐의 근육에서 MCD의 활성도는 대조군에 비해 rosiglitazone 을 투여한 군에서 2.4배, fenofibrate를 투여한 군에서는 1.8배 정도 증가하는 양상을 관찰하였으며, 고지방 식이를 섭취시킨 쥐의 근육에서는 rosiglitazone 과 fenofibrate 투여 한 군에서 대조군에 비해 각각 2.0배 와 4.5배 정도 증가하는 결과를 관찰 하였다. 그러나 AMPK 와 ACC 의 활성도는 정상 식이 와 고지방 식이를 섭취 시킨 근육조직에서

모두 의미 있는 차이를 관찰하지 못하였다. 심장조직에서 AMPK의 활성도는 fenofibrate를 투여한 군에서만 의미 있게 증가하였는데 정상 식이를 섭취한 군에서는 2.3배, 고지방 식이를 섭취 시킨 군에서는 1.7배 정도 증가하는 현상을 관찰 하였으며, 또한 고지방 식이를 섭취시킨 각 군에서 정상식이를 섭취시킨 각 군에 비해 현저히 증가하는 양상을 관찰 할 수 있었다. 정상 식이를 섭취시키고 fenofibrate를 투여한 군에서 MCD의 활성도는 30% 정도 증가하였으며, 고지방 식이를 섭취시키고 rosiglitazone 과 fenofibrate를 투여 한 두 군에서 각각 41% 정도 감소되는 현상을 관찰 하였다. ACC의 활성도는 모든 실험군에서 의미 있는 차이를 관찰하지 못하였다. 간 조직에서 AMPK의 활성도는 고지방 식이를 섭취시키고 fenofibrate를 투여 한 군에서만 72% 정도 감소 하였다. MCD의 활성도는 정상 식이를 섭취 시키고 fenofibrate를 투여한 군에서 2.7배 증가하였고 고지방 식이를 섭취 시키고 rosiglitazone 과 fenofibrate를 투여 한 군에서 각각 1.6배 와 2.0배 증가하는 양상을 관찰하였다. 고지방 식이를 섭취시킨 쥐의 간 조직에서 rosiglitazone 과 fenofibrate는 ACC의 활성도를 각각 38% 와 87% 정도 감소 시켰으며, 또한 fenofibrate는 rosiglitazone에 비해 ACC의 활성도를 79% 정도 감소시키는 현상을 관찰하였다. 정상 식이와 고지방 식이를 섭취한 실험군의 MCD mRNA 발현은 fenofibrate를 투여한 군에서만 의미 있게 증가하는 양상을 관찰할 수 있었다. 본 연구의 결과를 통해서, 제2형 당뇨병 모델인 OLETF 쥐의 당뇨병 전 단계에서 정상 식이 와 고지방 식이를 섭취 시키고 동시에 PPAR-γ 와 –α agonist 인 rosiglitazone 과 fenofibrate를 투여한 쥐의 근육, 심장, 간 등 조직에서 MCD 와 ACC 두 효소는 AMPK과 무관하게 조절 된다는 사실을 알 수 있었고, PPAR-γ agonist 인 rosiglitazone은 protein 단계에서 MCD를 조절하며 PPAR–α agonist 인 fenofibrate는 gene 단계에서부터 또는 gene 과 protein 두 단계에서 MCD를 조절 한다는 사실을 알 수 있었다.

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**핵심되는 말:** malonyl-CoA decarboxylase (MCD), acetyl-CoA carboxylase (ACC), AMP-activated protein kinase (AMPK), PPAR-γ and PPAR-α agonists, rosilitazone, fenofibrate, MCD mRNA