MATERIALS AND METHODS

Animal Care

The experiments were performed in male Wistar rats, weighing between 120-150 g. The animals were obtained from the National Animal Center, Salaya Campus, Mahidol University. All animals were housed individually in rodent cages in the animal room where the temperature was maintained approximately at 24-25 °C with 12:12 hours dark-light cycle. After being acclimatized for at least 5 days, the animals were matched as closely as possible for body weight and then randomly divided into 3 groups : normal fat diet (NF) , moderate fat diet (MF) and high fat diet (HF) groups. The animals in each group received diet with different fat content for 10 weeks. The NF group consumed a normal fat diet containing fat 11.34% of total energy in the diet (%E), protein 20.15% E and carbohydrate 68.5% E. The MF group consumed a moderate fat diet consisting of fat 20.14% E, protein 20.15% E and carbohydrate 59.44% E, while the HF group received a high fat diet containing fat 74.85% E, protein 20.15% E and carbohydrate 5% E. The composition of diets, based on caloric in normal diet, were calculated by modification from Conlee *et al.* (1990) (Table 1). Food and water were provided ad libitum and the food intake was daily recorded. All animals were simultaneously enrolled in the exercise training program during the period of dietary administration.

At the end of 10 weeks training, the animals were allowed to rest for 2 days. On the final day of the experiment, animals in each group were assigned to three subgroups: endurance, exercise and resting subgroups. The animals in endurance subgroup were subjected to endurance test and endurance times were determined. The animals in exercise subgroup received similar endurance test protocol and the animals in resting subgroup served as resting control. After that, all animals were immediately sacrificed for collection of blood and tissue samples.

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Table 1 Composition of experiment diets

Ingredients	NF		MF		HF	
	g	%kcal	g	%kcal	g	%kcal
Cornstarch	680	68.51	590	59.44	49.62	5
Lard	50	11.34	90	20.14	330.2	74.85
Casein	200	20.15	200	20.15	200	20.15
Vitamins and minerals	70	0.0	70	0.0	70	0.0

Diets ingredients and nutrient analyses were modified from Conlee *et al.*(1990). Energy (Kcal) per gram: carbohydrate 4; fat 9; protein 4. All diets were isocaloric except that the dietary fat contents increased in the order of NF < MF < HF. NF; normal fat diet, MF; moderate fat diet, HF; high fat diet.

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Exercise Procedure

The exercise training program was modified from the training protocol of Mokelke *et al.* (1997). All animals were trained on a motorized rodent treadmill (Columbus instrument, USA) five days per week for a period of 10 weeks. The animal started treadmill running at a speed of 20 m/min for 3 weeks and was increased to 24 m/min in the 4th week and then to the final speed of 28 m/min after 6 weeks. The running grade was 5% for the first 3 weeks and was increased to 10% at the 5th week. The running time was 10 min for the 1st week and was increased by 10 min every week until the maximal running duration was 60 min. Therefore, the animal was running at a speed of 28 m/min, 10% grad, 60 min/day, 5 days/week from week 7 until the end of the experiment.

Endurance Test

The endurance test protocol was modified from the method of Mercier *et al.* (1995) and Miller *et al.* (1984). Rats ran on the treadmill with speed of 30 m/min at 10% grade until they could no longer keep pace with the treadmill or they came in contact with electric grid at the rear of the treadmill five times in two minutes. At that point, exhaustion time of the exercise was determined. All rats ran under the same investigator to avoid any variation in judgement of exhaustion that might exist among investigators.

Exercise Test

To investigate the effects of dietary fat on the energy metabolism during the initial stage of exercise, the exercise test protocol was used in the study. The fundamental procedure of the exercise test was similar to endurance test protocol except for the duration of exercise. The speed of treadmill was 30 m/min at 10% grade and the duration of exercise running in each subgroup was only 1/3 of the individual average endurance time. As a consequence, all rats received approximately the same relative exercise intensity.

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Animal Sacrifice and Tissue Preparation

At the time of tissue collection, animals were killed after being anesthetized with Pentobarbital sodium (50 mg/ kg body weight) administered intraperitoneally. Under anesthesia, both hindlegs were skinned and samples of the soleus and vastus lateralis muscles were surgically removed from each leg. On removal, each muscle sample was trimmed of connective tissue and adherent adipose tissue and frozen in liquid nitrogen. Thereafter, the abdominal cavity was opened up to the diaphragm and one lobe of liver was excised and similarly frozen. All tissue was stored at –70 °C until time for determination of enzyme activity, glycogen and triacylglycerol content. After tissue excision, blood was drawn from the heart and placed into glass tubes containing sodium fluoride and potassium oxalate anticoagulant. The blood sample was then mixed and centrifuged. The plasma was separated and stored at –70 °C until time for assay. All sacrifice and exercise procedures were performed at the same time of day to minimize diurnal glycogen variation.

Biochemical Analysis

Determination of Lipid Profiles and Lactate

Measurement of lipid profiles and blood lactate concentration were achieved by the facility from Central Diagnostic Laboratory, Maharaj Nakorn Chiangmai Hospital. The plasma cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and lactate were assayed by enzymatic methods using commercial kits (Cholesterol *Des*, Erba Diagnostics Mannheim, Germany; Wako L-Type LDL-C & Wako L-Type HDL-C, Wako Pure Chemical Industries, Ltd., Japan; Lactate PAP, bioMerieux sa, France), adapted for use on automatic analyzer (Merck Co. Ltd., Germany).

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Determination of Glucose

The principle of the enzymatic colorimetric method is based on the fact that glucose in the plasma sample is oxidized by the glucose oxidase into the gluconate and hydrogen peroxide (H_2O_2). The amount of H_2O_2 formed is proportional to the glucose content of the sample. In the

presence of peroxidase, H_2O_2 reacts with a substrate chromogen to form p-chlorophenol (red-chinonimin). The absorbance of the reaction mixture is read with a spectrophotometer.

The plasma glucose concentration was determined by enzymatic colorimetric method using commercial kits (Biosub GLU, Biocon Diagnostik, Germany). The absorbance was read with a spectrophotometer (Helios Delta, ThermoSpectronic, England) at 546 nm against the blank. A standard curve of glucose concentration was constructed by plotting the absorbances against the respective concentrations of standard glucose. The glucose concentration in each unknown sample was read out by interpolation of its absorbance on the standard curve.

Determination of Triacylglycerol and Glycerol

The principle of the enzymatic colorimetric method is that the triacylglycerol in the plasma sample is hydrolized by the lipoprotein lipase into glycerol and free fatty acids. In the presence of ATP and glycerolkinase, glycerol forms glycerol-3-phosphate which is further oxidized by glycerol-3-P-oxidase into DAP and H_2O_2. The enzyme peroxidase in the substrate-chromogen solution catalyses the reaction between H_2O_2, p-chlorophenol and 4-aminoantipyrine to form the color complex (quinonimine). The absorbance of the reaction mixture was read with a spectrophotometer.

The plasma triacylglycerol or glycerol concentration was analyzed using a commercial enzymatic colorimetric (Fluitest TG, Biocon Diagnostik, Germany). The absorbance was read with a spectrophotometer (Helios Delta, ThermoSpectronic, England) at 546 nm against the blank. A standard curve of glycerol concentration was constructed by plotting the absorbances against the respective concentrations of standard glycerol. The triacylglycerol or glycerol concentration in each unknown sample was read out by interpolation of its absorbance on standard curve.

Determination of Tissue Glycogen Content

The glycogen was determined as glucose residues after hydrolysis by amylo-α-1, 4-α1, 6-glucosidase (AG) enzyme. Tissue homogenates were prepared for glycogen assay by a modification of the method of Passonneau and Lauderdale (1974). A 100-200 mg portion of liver and muscle was minced and placed in 3-5 ml of 0.03 N HCl. The homogenate was placed in a boiling water bath for 5 min and was centrifuged at 4,000 rpm for 15 minutes to obtain clear

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supernatant. A 150 μl of supernatant was added into a glass tube containing 150 μl of 0.4 M acetate buffer (0.2 M acetic acid: 0.2 M sodium acetate, pH 4.7) and 50 μl of AG. The reagent was then mixed and incubated at 37 °C in a water bath for 2 hours. After that, the glucose formed was determined by enzymatic colorimetric method using commercial kits (Biosub GLU, Biocon Diagnostik, Germany).

The tissue glycogen utilization rate was calculated as follow:

Average rate of tissue glycogen utilization (μg/gm tissue/min)

$$= \frac{\text{(Resting tissue glycogen -- Tissue glycogen after exercise test or exhaustion)}}{\text{Duration of exercise or Endurance time}}$$

Determination of Tissue Triacylglycerol content

Tissue homogenates were prepared for triacylglycerol assay by a modification of the method of Frayn and Maycock (1980). A 100-200 mg portion of liver and muscle was minced and put into a glass tube containing 3 ml of chloroform-isopropanol 7:11 (v/v). The homogenate was left at room temperature for at least 16 hours. Then, a 1 ml of homogenate was pipetted into a glass tube and evaporated to dryness at 40 °C for 16 hours. The dried residue was dissolved and mixed in 10% bovine serum albumin. The triacylglycerol concentration was analyzed with a commercial enzymatic colorimetric (Fluitest TG, Biocon Diagnostik, Germany).

The tissue triacylglycerol utilization rate was calculated as follow:

Average rate of tissue triacylglycerol utilization (μg/gm tissue/min)

$$= \frac{\text{(Resting tissue triacylglycerol -- Tissue triacylglycerol after exercise test or exhaustion)}}{\text{Duration of exercise or Endurance time}}$$

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Determination of Muscle Enzyme Activity

A. Preparation of Tissue Homogenate

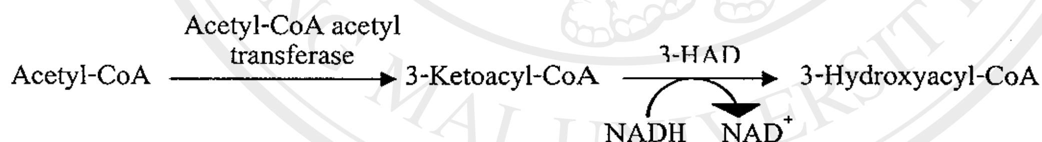
The homogenate preparation was done following the method described by Campbell and Febbraio (2001) and Krieger *et al.* (1980). The entire procedures were performed at 0-4 °C. A 250-300 mg portion of muscle was finely minced and placed in 3 ml of reagent (100 mM KCl, 10mM Tris, 5 mM Na EDTA, and 1% Triton X, pH 7.4) then centrifuged at 10,000 rpm for 10

minutes by a centrifuge (Sorval RC-5 superspeed Refrigerated Centrifuge, Germany). The supernatant was removed and stored in -70 °C for subsequent enzymatic analysis.

B. Determination of 3-Hydroxyacyl CoA Dehydrogenase (3-HAD) Activity

According to the method of Bass *et al.* (1969) and Swislocki *et al.* (1999), the 3-HAD activity was monitored by following the disappearance of β-NADH [beta-nicotinamide adenine dinucleotide (reduced form)] when using acetyl-CoA as substrate. A 60 μl of supernatant was added into a cuvette containing 1.2 ml of reaction mixture (40 mM HEPES, 1 mM NaN_3, 5 mM EDTA, 0.45 mM NADH and 0.1 mM acetoacetyl-CoA , pH 7.4). The disappearance of β-NADH was monitored by measuring the absorbance at wavelengths 340 nm using spectrophotometer (Helios Delta, ThermoSpectronic, England). The absorbance was followed every 30 seconds by using kinetic program in the spectrophotometer. Linear rates were obtained for at least 3 minutes. The baseline rate of the disappearance of β-NADH was subtracted from the rate after addition of supernatant into reactive mixture. A molar extinction coefficient of 6,200 M^{-1}/cm for β-NADH was used for calculations.

The steps of reaction are schematically presented as followed :



C. Determination of Citrate Synthase (CS) Activity

According to the method of Srere (1969), the CS activity was determined by measuring the appearance of the free SH group released from the CoASH using 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB). A 0.06 ml of supernatant and 0.924 ml of H_2O was put into a cuvette containing 0.12 ml of DTNB, 0.036 ml of acetyl-CoA. The citrate synthase reaction was started by the addition of 0.06 ml of oxaloacetate. The appearance of free SH group released from the CoASH was monitored by measuring the absorbance at wavelengths at 412 nm. The absorbance was followed every 30 seconds to measure possible citrate synthase activity. Linear rates were

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obtained for at least 3 minutes. A molar extinction coefficient of 13,600 M^{-1}/cm for Co A was used for calculations.

The steps of reaction are schematically presented as followed :

Statistical Analysis

All values are expressed as mean + SE. The significance of difference among experimental groups was determined using one way analysis of variance (ANOVA). The LSD post hoc test was used to identify specific mean differences. For all statistical analysis, a level of $p < 0.05$, 0.01 was considered to be significant difference.

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