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Effect of Silymarin on Gluconeogenesis and Lactate Production in Exercising Rats

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Abstract In this study, we investigated the effects of silymarin (SM) on gluconeogenesis during exercise in rats. After 4 weeks of exercise, blood samples, liver, and skeletal muscle tissues were collected, and the levels of triglycerides (TG), lactate, peroxisome proliferator activated receptor gamma (PPARγ), phosphoenol pyruvate carboxykinase (PEPCK), pyruvate dehydrogenase kinase 4 (PDK4), and phosphorylated 5-AMP activated protein kinase (AMPK) were measured. The TG and lactate level of the serum were reduced. In addition, the expression of Akt, PEPCK, and PPARγ in liver was decreased as well as the expression of AMPK in muscle. On the contrary, the level of PDK4 in muscle was increased. These results showed that that administration of SM ameliorated exercise-induced gluconeogenesis and β-oxidation through the regulation of PPARγ, PEPCK, and PDK4. Thus, intake of SM during exercise may improve endurance by modulating of the metabolism of glucose, lipids, and lactate.

Keywords: silymarin, exercise, gluconeogenesis, β-oxidation

Introduction

During exercise, the liver is the central organ responsible for the control of glucose homeostasis (1). The adaptation of the liver to exercise involves the enhanced expression of the gluconeogenic enzyme, phosphoenol pyruvate carboxykinase (PEPCK) (2,3). This enzyme is directly associated with blood glucose homeostasis and catalyzes the first rate-limiting step of gluconeogenesis. Other signaling molecules, including peroxisome proliferator activated receptors (PPARs) and AMP-activated kinase (AMPK), also regulate gluconeogenesis and fatty acid oxidation in the liver and muscles. In particular, AMPK plays a key role in the activation of fat oxidation and glucose uptake in skeletal muscle and acts as an intracellular fuel sensor (4). Changes in the cellular energy state are likely to modulate the activity of AMPK and the glucose demand (5).

Pyruvate dehydrogenase kinase 4 (PDK4) is regulated by several physiological conditions and controls the activity of the pyruvate dehydrogenase complex (PDC), which plays a pivotal role in glucose oxidation in mitochondria (6,7). The expression of PDK4 is increased in several tissues, including the heart, kidney, skeletal muscle, and adipocytes, in response to starvation and type 2 diabetes (8,9). Levels of glucocorticoids and PPARα may be altered in response to induction of PDK4 expression in both starvation and diabetic models (10). However, the role of PDK4 during exercise has not been examined.

Low-intensity exercise is thought to be the most effective form of exercise to increase physical activity (11). Moreover, regular exercise has beneficial effects on the liver and muscle. Recently, many attempts have been made to improve physical performance using nutritional approaches given that nutrients provide energy and regulate physiological processes associated with exercise (12). Silymarin (SM), an active flavonoid component in milk thistle, has been used to alleviate symptoms in patients suffering from liver diseases and is well known for its antioxidant and anticancer effects (13). Furthermore, SM has been used clinically as an adjuvant in the treatment of alcoholic liver disease; however, the effects of SM during exercise have not been evaluated.

Therefore, we examined the effects of SM on lipid and glucose metabolism during exercise. Our results provide important insights into the contribution of SM to the enhancement of physical strength and endurance.

Materials and Methods

Animal care Inbred male Sprague-Dawley rats, 6 weeks of age with

an average body weight of 195±10 g, were purchased from Dae-Han Experimental Animal Center (Eumsung, Korea). Rats were acclimatized for 1 week in a housing facility maintained at 23°C with a 12 h/12 h light/dark cycle, and randomly assigned to treatment groups. The rats were housed four/cage in polycarbonate shoe box-style cages lined with Harlan Teklad Laboratory Grade Sani-chips (Harlan Laboratories, Indianapolis, IN, USA), which was changed every other day. Rodent chow (Superfeed Co., Ltd., Wonju, Korea) and purified water (Milli-Q Water Purification System; EMD Millipore, Billerica, MA, USA) were supplied *ad libitum*. All procedures involving the use of animals were approved by the Institutional Care and Use Committee and carried out in accordance with the guidelines established by the Public Health Service Policy on the Human Care and Use of Laboratory Animals (KU14012).

Experimental protocol (−)-Epigallocatechin 3-*O*-gallate (EGCG) and SM were purchased from Sigma-Aldrich (St. Louis, MO, USA). Slide kits for glucose, triglyceride, and lactate measurement were procured from Ortho Clinical Diagnostics Inc. (Vitros-350; Rochester, NY, USA). All rats have free access to food and water for 4 weeks and were then divided into one sedentary and three exercise-training groups (*n*=8/group). Rats in the exercise-training groups ran on a motor-driven treadmill (Muromachi Kikai Co., Ltd., Tokyo, Japan) for 60 min/day, 5 days/week. The running speed was 8 m/min, which has been defined as exercise (14). Immediately after the end of exercise on the last day, the rats were starved for 12 h to eliminate any confounding effects of the last meal. The rats were then sacrificed between 09:00 and 10:00 AM. Arteriovenous blood was collected under CO_2 anesthesia, following which, the abdominal cavity was opened and the liver quickly excised, washed, weighed, and frozen at −80°C for further analysis.

Serum levels of glucose, triglyceride, and lactate The serum levels of glucose, triglyceride, and lactate in fed rats were measured weekly. The sedentary group (control) was fed carrier, and the three exercise-trained (EX) groups were fed carrier, EGCG, and SM, respectively. EGCG and SM were dissolved in DMSO (0.1%) and administered by gavage at dosages of 50 mg/kg/day (EGCG+EX and SM+EX, *n*=8) for 4 weeks during the exercise period. The blood samples drawn at before- and post-exercise from tail were centrifuged at 10,000x*g* for 15 min and the serum was stored at −80°C until analysis.

Western blot analysis The liver and skeletal muscle were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, 10 mg/mL of leupeptin, 10 mg/mL of pepstatin A, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride). Tissue lysates were centrifuged at 12,000×*g* for 20 min at 4°C, and the supernatant was mixed with one-fourth the volume of 4x SDS sample buffer, boiled for 5 min, and then separated on a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nylon membrane by means of Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). The western blot analysis was carried out as described elsewhere (15). The bands were visualized by ECLTM western blotting detection reagents (GE Healthcare, Little Chalfont, UK) and exposed to X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR) Semi-quantitative RT-PCR was performed to determine the expression levels of each gene of interest. Each set of samples was analyzed in triplicate. The conditions for RT-PCR were similar to ones previously described (16). Reverse transcription of the RNA was performed using the RT-PreMix Kit (iNtRON Biotechnology, Sungnam, Korea). One microgram of RNA and 20 pmol primers were preincubated at 70°C for 5 min and transferred to a mixture tube. cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 94°C for 5 min. Thereafter, the RT-generated DNA was amplified using the PCR PreMix Kit. The primers used for cDNA amplification were: 5'-TTGCCATCGAAGGCATCA-3' (sense) and 5'-TCTCATGGCAGCTCCTACA AACAC-3' (antisense) for PEPCK; 5'-AACGTGTGTCTGTCCCGGATCTAC-3' (sense), 5'-ACCTCTGGAGGCTGGCATTG-3' (antisense) for G6Pase; 5'-CATTTCTGCTCCACACTATGAA-3' (sense), 5'-CGGGAAGGACTTTATG TATGAG-3' (antisense) for PPARγ; and 5'-CGGAGTCAACGGATTTGG TCGTAT-3' (sense), 5'-AGCTTCTCCATGGTGGTGAAGAC-3' (antisense) for GAPDH. The expected PCR products were 126 (for PEPCK), 73 (for G6Pase), 550 (for PPARγ), and 306 bp (for GAPDH). PCR products were subjected to electrophoresis on a 1.2% agarose gel and stained with ethidium bromide.

Statistical analysis Statistical analyses were performed using SAS statistical software (SAS institute, Cary, NC, USA). Treatment effects were analyzed using an ANOVA followed by Dunnett's multiple range tests. Significance was set at $p<0.05$.

Results and Discussion

Analysis of body and liver weight Figure 1 shows changes in body and liver weights following oral administration of EGCG and SM during exercise. As expected, body weight was significantly lowered in all EX group compared with that in the control group (Fig. 1A). However, there was no difference in body weight in the SM+EX and EGCG+EX groups compared with that in the EX control group. Furthermore, liver weights were not significantly different among the study groups (Fig. 1B).

Serum analysis Serum levels of triglycerides, lactate, and glucose were measured before and after exercise (Fig. 2). As shown in Fig. 2A, triglyceride levels were significantly lower after exercise than before exercise in all EX groups. EGCG (50 mg/kg BW) further lowered

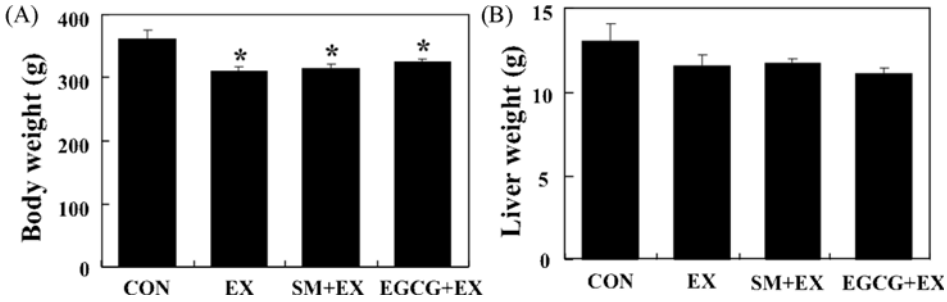

Fig. 1. Changes in body (A) and liver weights (B) in rats. Protocols for exercise training and EGCG (50 mg/kg/day) and SM (50 mg/kg/day) administration are described in the Materials and Methods. The results are expressed as means±SE (*n*=8). *Significantly different from the control group at $p<0.05$. EX, exercise group; EGCG, (–)-Epigallocatechin 3-*O*-gallate; SM, silymarin

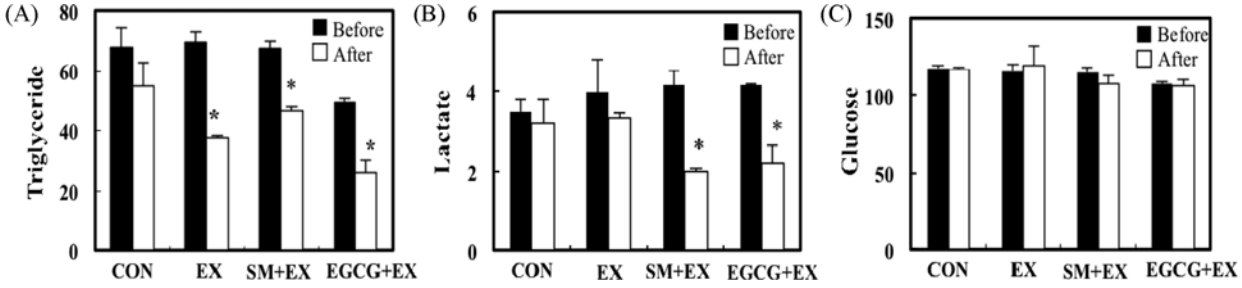

Fig. 2. Serum levels of triglycerides (A), lactate (B), and glucose (C) before and after exercise. Protocols for exercise training and EGCG (50 mg/kg/days) and SM (50 mg/kg/days) administration have been described in the Materials and Methods. Results have been expressed as means±SE (*n*=8). *Significantly different than the control group at $p<0.05$. EX, exercise group; EGCG, (–)-Epigallocatechin 3-*O*-gallate; SM, silymarin

serum triglyceride levels compared with that in rats subjected to exercise alone (Fig. 2A). Basal lactate levels were not significantly different among the experimental groups. After exercise, lactate levels of the control and EX alone groups were not altered; however, lactate levels in the SM+EX and EGCG+EX groups were significantly lower after exercise than before exercise (Fig. 2B). Interestingly, serum glucose levels were not altered after exercise in the experimental groups (Fig. 2C). Furthermore, neither SM nor EGCG altered serum glucose levels (Fig. 2C). We assume that the glucose level would be shifted in diabetic rats, however our experiments were conducted under normal rats system, so there might be no changes.

Modulation of gene expression Next, we investigated the mechanisms through which liver glucose metabolism was modulated by exercise and/or flavonoids by measuring the phosphorylation of Akt, an important component of the insulin signaling pathway, using western blotting. The phosphorylation state of Akt from EX, SM+EE, and EGCG+EX groups was significantly lower than that of the control (Fig. 3A). Neither SM nor EGCG altered Akt phosphorylation in the liver compared with that in the exercise alone group (Fig. 3A). These results indicated that insulin signaling was suppressed during exercise in order to increase gluconeogenesis from lactate produced during anaerobic glycolysis in skeletal muscle.

In order to elucidate the mechanisms through which blood glucose levels were maintained in the SM+EX and EGCG+EX groups despite changes in lactate levels, the gene expression levels of PEPCK and PPARγ, which are both involved in gluconeogenesis and β-oxidation, were examined in the livers by RT-PCR. Exercise alone induced the expression of PPARγ and PEPCK in the liver. In addition, the expression levels of PEPCK and PPARγ were significantly upregulated in the livers of rats in the SM+EX and EGCG+EX groups after exercise as compared with those in rats subjected to exercise alone (Fig. 3B, 3C, and 3D). This suggested that hepatic glucose production might be increased in the livers of SM+EX and EGCG+EX groups.

Phosphorylation of Akt plays a major role in insulin-stimulated glucose uptake in skeletal muscles. Thus, we next examined the effects of SM plus exercise on glucose uptake and the energy state in the gastrocnemius muscle (GA) and soleus muscle (SOL) by measuring the phosphorylation of Akt and AMPK in skeletal muscles immediately after exercise. The results indicated that Akt phosphorylation was dramatically increased by exercise in the SOL but not the GA. However, the basal phosphorylation of Akt was higher in the GA than in the SOL (Fig. 4A and 4C). The phosphorylation of AMPK was increased by exercise in the GA and SOL, indicating that the cellular energy state was decreased by exercise in the skeletal muscles. Additionally, AMPK phosphorylation was decreased in the SM+EX and EGCG+EX groups for both muscle types (Fig. 4B and 4D).

The expression level of PDK4 is increased during exercise, resulting in increased fat oxidation in order to meet energy demands during exercise (17,18). Interestingly, PDK4 expression was not increased in the GA after exercise (Fig. 4E); however its expression was significantly

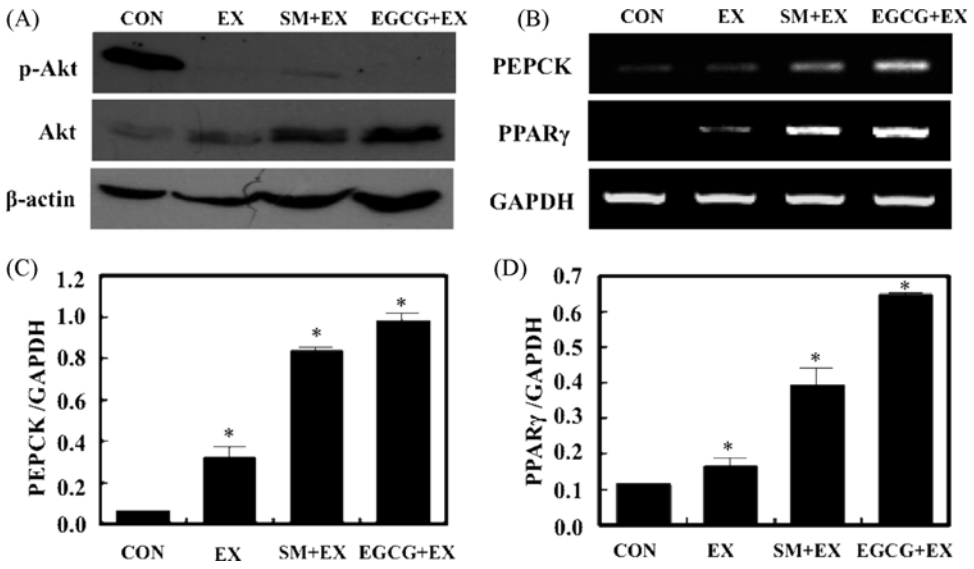

Fig. 3. Effect of silymarin on the phosphorylation of Akt and hepatic gene expression in the liver. (A): Proteins obtained from rat liver were separated by SDS-PAGE on 12% gels and transferred to nylon membranes. Western blot analysis was carried out with anti-phospho-Akt and anti-total Akt antibodies by conventional methods. EX: exercise group, EGCG: (–)-Epigallocatechin 3-*O*-gallate, SM: silymarin. (B), (C), and (D): The hepatic expression of genes in the rats given SM for four weeks was determined by RT-PCR. Products were electrophoresed on a 1.5% agarose gel, visualized by staining with ethidium bromide, and certificated using a digital camera. The expression level of PEPCK and PPARγ was digitized using UN-SCAN-IT software. Data represents the means±SE of two independent experiments. *Significantly different than the control group at $p<0.05$. EX, exercise group; EGCG, (–)-Epigallocatechin 3-*O*-gallate; SM, silymarin

increased in the SOL (Fig. 4F). Administration of SM and EGCG increased PDK4 expression in both the GA and SOL.

SM is a flavonoid that may have biological effects similar to those of phytochemicals such as EGCG, which is found in green tea. Green tea has been shown to exert beneficial effects by increasing the expression of glucose transporter 4 in adipose tissues, leading to increased glucose uptake into adipose tissues and ameliorating symptoms of metabolic syndrome, such as insulin resistance and hypertension (19). In addition, EGCG supplementation has been shown to cause a pronounced decrease in glucose levels by decreasing hepatic glucose output and increasing glucose-stimulated insulin secretion (20). Similarly, SM has been shown to reduce serum glucose levels in diabetic rats by modulating hepatic mitochondrial function (21). These reports suggest that SM may protect against various types of injury induced by exercise, leading to enhanced physical activity and strength. Therefore, in this study, we examined, for the first time, the effects of SM administration on hepatic gluconeogenesis and lactate production in the skeletal muscles of exercising rats. Our findings clearly showed that SM had beneficial effects similar to exercise endurance in exercise-trained rats. Thus, SM may represent a novel supplement for improving physical performance and activity.

Endurance exercise training has been shown to induce physiological adaptation and increase aerobic energy metabolism (22). Moreover, exercise training can decrease body weight and serum levels of triglycerides and lactate (21). Consistent with these previous findings, we showed that exercise reduced body weight and serum triglycerides in trained rats but did not alter serum levels of lactate or glucose. Notably, the EX groups did not show altered serum lactate levels, whereas administration of SM or EGCG with exercise significantly reduced serum lactate levels. A previous study reported that serum lactate and triglycerides are reduced by EGCG in exercised mice (23). Generally, serum lactate is increased during exercise because of limited oxygen supply, which leads to muscle fatigue. Therefore, the observed decreases in serum lactate by SM administration during exercise indicated that SM administration improved exercise endurance due to reduced accumulation of lactate in skeletal muscles. The lactate produced from skeletal muscle may be used for hepatic gluconeogenesis to provide glucose to peripheral tissues during exercise (24). Indeed, blood glucose levels were not decreased during exercise, although glucose uptake was presumably increased in skeletal muscles because Akt phosphorylation was dramatically increased in the SOL of rats in the EX groups.

Instead of glucose oxidation, fat oxidation has been shown to increase significantly in order to provide energy during exercise (25). Murase *et al.* reported that plasma lactate concentrations in mice fed green tea extracts were significantly lower after exercise, concomitant with increases in serum free fatty acids and β-oxidation activity in skeletal muscle (23), suggesting that EGCG may increase fat oxidation in skeletal muscle. In our study, AMPK phosphorylation in skeletal muscles obtained from EGCG- or SM-treated rats was significantly lower than that of exercise-trained rats, indicating that there was no limit to the supply of ATP to the muscle. Presumably, in these rats, ATP supplementation may occur through fat oxidation and not

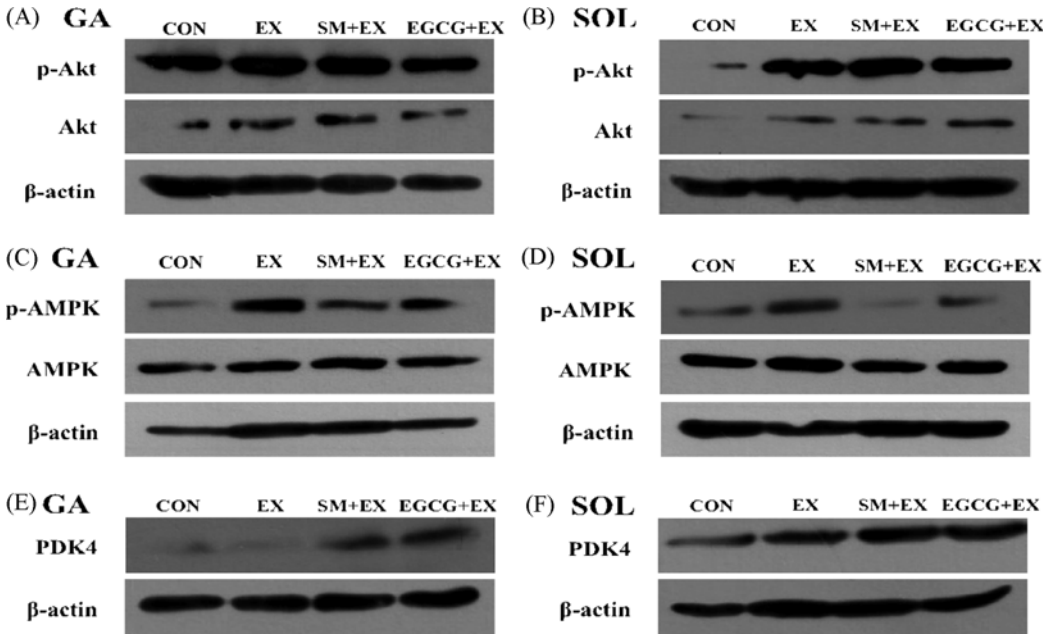

Fig. 4. Effects of silymarin on the phosphorylation of Akt and AMPK in muscle and the expression of PDK4 in the gasterocnemius and soleus muscle. (A) and (B): Proteins obtained from gastrocnemius muscle. (C), (D), (E), and (F): Proteins obtained from gastrocnemius muscle and soleus muscle. GA, gastrocnemius muscle; SOL, soleus muscle; EX, exercise group; EGCG, (–)-Epigallocatechin 3-*O*-gallate; SM, silymarin

glucose oxidation because PDK4 expression was significantly increased in the skeletal muscles obtained from SM- or EGCG-treated rats. Consistent with this, PDK4 expression has been shown to be increased during exercise (17,26); this may result in increased β-oxidation rather than glucose oxidation, indicating that induction of PDK4 may regulate the fuel selection between glucose and fatty acids during exercise, depending on the physiological demand (18,27,28). Exercise-induced PDK4 inhibits glucose oxidation via the phosphorylation of PDC, which results in suppression of PDC activity (27). However, the molecular mechanisms through which PDK4 expression is induced during exercise are not yet clear. In our previous study, we showed that PDK4 expression was induced by glucocorticoids, PPARs, and insulin resistance (8,29). Moreover, in the current study, PDK4 expression was significantly induced by SM, EGCG, and exercise in the skeletal muscles, which may be explained, at least in part, by PPAR activation. Although the exact mechanism is not clear, EGCG has been shown to increase the expression levels of PPARs in adipose tissues and cardiomyocytes (30,31). In this study, we also found that EGCG and SM supplementation increased the expression of PPAR in the liver, suggesting that PPAR induction can mediate PDK4 upregulation in the skeletal muscle.

Insulin significantly represses PDK4 induced by starvation and diabetes in the liver, kidney, and skeletal muscle (9). In the present study, exercise significantly reduced the phosphorylation of Akt, and treatment with SM or EGCG further decreased Akt phosphorylation in liver. PEPCK, which is downregulated by insulin (31), catalyzes the first step of hepatic gluconeogenesis, and the observed increase in PEPCK expression in the livers of rats treated with SM or EGCG plus

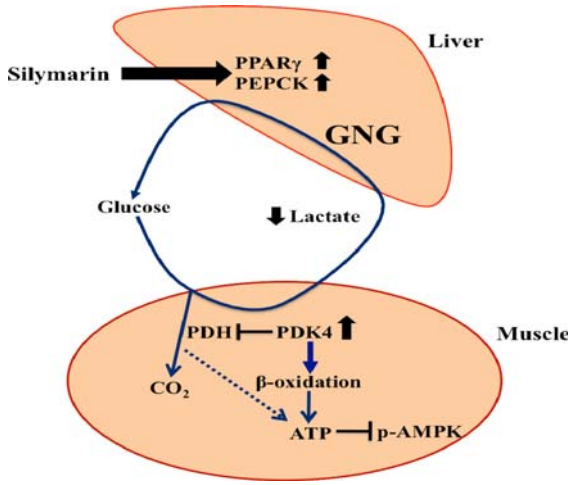

Fig. 5. Schematic diagram of the effect of silymarin on hepatic gluconeogenesis and metabolic regulation of glucose and fat in the skeletal muscle. Silymarin increased the expression of gluconeogenic genes in the liver, thereby increases consumption of serum lactate. Increased levels of PDK4 induced by silymarin in skeletal muscle inhibited PDH activity to conserve lactate for gluconeogenesis and may increase the oxidation of free fatty acids to supply ATP in the muscle, resulting in increased muscle movement and inhibition of phosphorylation of AMPK. GNG, gluconeogenesis

exercise indicated that gluconeogenic capacity in treated rats was greater than that in untreated rats, resulting in lower lactate levels and maintenance of glucose levels in serum. However, Akt phosphorylation in skeletal muscle was dramatically increased after exercise, indicating that insulin-dependent glucose uptake was

significantly increased in the skeletal muscles in order to supply energy during exercise. This may have functioned to maintain the euglycemic state in SM- and EGCG-treated rats, blocking increased hepatic glucose production. A possible pathway for the effects of SM on hepatic gluconeogenesis and the metabolic regulation of glucose and fat in the skeletal muscle based on our study is presented in the schematic in Fig. 5.

In summary, we showed that long-term treatment with SM combined with low-intensity exercise increased the utilization of lactate for gluconeogenesis via induction of the PPARγ-PEPCK signaling pathway in the liver, resulting in lower lactate levels and triglyceride in the blood. Induction of PDK4 in skeletal muscle may respond to increased fat oxidation in order to supply ATP for muscle contraction. The results of this study support the beneficial effects of SM plus exercise on the amelioration of metabolic syndrome. However, further studies are needed to better understand how SM induces the expression of PPARγ.

Disclosure The authors declare no conflict of interest.

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