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Monascus-Fermented Yellow Pigments Monascin and Ankaflavin Showed Antiobesity Effect via the Suppression of Differentiation and Lipogenesis in Obese Rats Fed a High-Fat Diet

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ABSTRACT: *Monascus*-fermented monascin and ankaflavin are found to strongly inhibit differentiation and lipogenesis and stimulate lipolysis effects in a 3T3-L1 preadipocyte model, but the *in vivo* regulation mechanism is unclear. This study uses obese rats caused by a high-fat diet to examine the effects of daily monascin and ankaflavin feeding (8 weeks) on antiobesity effects and modulation of differentiation, lipogenesis, and lipid absorption. The results show that monascin and ankaflavin had a significant antiobesity effect, which should result from the modulation of monascin and ankaflavin on the inhibition of differentiation by inhibiting CCAT/enhancer-binding protein β (C/EBPβ) expression (36.4% and 48.3%) and its downstream peroxisome proliferator-activated receptor γ (PPARγ) (55.6% and 64.5%) and CCAT/enhancer-binding protein α (C/EBPα) expressions (25.2% and 33.2%) and the inhibition of lipogenesis by increasing lipase activity (14.0% and 10.7%) and decreasing heparin releasable lipoprotein lipase (HR-LPL) activity (34.8% and 30.5%). Furthermore, monascin and ankaflavin are the first agents found to suppress Niemann-Pick C1 Like 1 (NPC1L1) protein expression (73.6% and 26.1%) associated with small intestine tissue lipid absorption. Importantly, monascin and ankaflavin are not like monacolin K, which increases creatine phosphokinase (CPK) activity, known as a rhabdomyolysis indicator.

KEYWORDS: *obesity, Monascus, monascin, ankaflavin, monacolin K*

INTRODUCTION

Obesity is associated with a higher risk of developing diabetes and cardiovascular disease. At the cellular level, enlargement of the adipose tissue mass has been characterized by an increase in the size (hypertrophy) or number (hyperplasia) of adipocytes. The triglyceride (TG) content in adipocytes reflects the balance between lipogenesis and lipolysis, which is largely related to cell volume. When adipocytes reach a critical size threshold, preadipocytes in close proximity to the adipocytes will respond to positive energy balance by proliferating and then differentiating into adipocytes to store the excess energy.[1] Early in life, adipose tissue expansion occurs primarily through hyperplasia. However, humans and rodents have the capacity to form new fat cells from preadipocytes throughout life. Several mechanisms reduce the risk of obesity, including reduced food intake, decreased intestine adsorption, suppressed lipogenesis, enhanced lipolysis and fatty acid oxidation, increased energy expenditure, and inhibited preadipocyte proliferation and differentiation.[2,3]

A *Monascus* species has been used as the traditional food fungus in Eastern Asia for several centuries. *Monascus*-fermented products are gradually developing as popular functional foods for the prevention of cardiovascular disease. In addition to cardiovascular disease, the *Monascus*-fermented product of red mold rice (RMR) is further proven to prevent obesity development via the suppressions of differentiation and lipogenesis in many previous *in vitro* and *in vivo* studies.[4,5] Although monacolin K is suggested as one of the functional components in RMR, its effect is less than the antiobesity effect of RMR. Therefore, after a screening test, monascin and ankaflavin were isolated from RMR and found to repress the expression of transcription factors during differentiation of 3T3-L1 preadipocytes.[6]

Monascin and ankaflavin are both yellow pigments consisting of azaphilonoid structure.[7] Monascin, known as an anti-inflammation agent, has been proven to protect the liver from chemical damage.[7] Our previous studies have confirmed that *Monascus*-fermented secondary metabolite yellow pigments monascin and ankaflavin reduce serum total cholesterol (TC), triglycerides (TG), and low density lipoprotein cholesterol (LDL-C) levels and increase high density lipoprotein cholesterol (HDL-C) levels to reduce blood lipids.[8] The *in vitro* results have preliminarily shown that monascin and ankaflavin inhibit 3T3-L1 preadipocyte differentiation, stimulate mature cells to break down lipids in the cell and release glycerol, and reduce mature adipocyte heparin releasable lipoprotein lipase (HR-LPL) activity, thereby lowering TG synthesis and accumulation in cells.[6]

However, there have not been any animal research experiments investigating the effects of monascin and ankaflavin on factors related to the weight, body fat, lipid absorption, and

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adipogenesis of rats suffering from obesity caused by a high-fat diet. This study uses an animal model of rats with obesity caused by a high fat diet to examine the effects of daily monascin and ankaflavin feeding on obesity factors including weight gain, food intake, food efficiency, fat pad weight, body fat percentage, adipocyte number, cell cross-sectional area, lipolysis activity, HR-LPL activity, and the expression of transcription factors related to adipose tissue (peroxisome proliferator-activated receptor γ (PPARγ), CCAT/enhancer-binding protein α (C/EBPα), and CCAT/enhancer-binding protein β (C/EBPβ)). Monacolin K produced by *Monascus* species is also proven to contribute to the antiobesity effect of RMR via the modulation of the proliferation, differentiation, and lipolysis in the adipocytes. Therefore, monacolin K is used as the positive control group and effective comparison group in this study.[4] However, the doses of monascin, ankaflavin, and monacolin K are used according to the contents in RMR in order to understand the contribution of the three compounds to the antiobesity effect.

MATERIALS AND METHODS

Chemicals. Monacolin K (mevanolin) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). LC grade acetonitrile, chloroform, methanol, and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar (PDA), and Bacto-agar were purchased from Difco Co. (Detroit, MI, USA). Monoclonal C/EBPα antibody was purchased from GeneTex Co (Irvine, CA, USA). Monoclonal C/EBPβ antibody and polyclonal PPARγ antibody were purchased from Novus Biological (Littleton, CO, USA). Monoclonal microsomal triglyceride transfer protein (MTP) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Monascin and Ankaflavin Purification. Monascin and ankaflavin (99.9% purity) were purified from RMR fermented by *Monascus purpuresus* NTU 568 according to the method in our previous study.[9]

Animal Experiments. The animal experiments' protocol refers to our previous study involving the antiobesity evaluation of RMR.[4] Male Sprague−Dawley (SD) rats at 6−8 weeks of age were purchased from BioLasco Co. (Taipei, Taiwan). The animals were housed individually and allowed free access to a standard laboratory chow (Ralston Purina, St Louis, MO, USA) and water. Three weeks later, the rats were randomly assigned to one of the following diets for 8 weeks: standard chow (control group, NOR; 4.5% fat, 3.34 kcal/g), high-fat and high-cholesterol diet consisting of 26.7% butter powder (Gene Asia Biotech Co., Ltd., Nang-Tou, Taiwan) in standard chow (HFC group; 30% fat, 0.2% cholesterol, 4.85 kcal/g), HFC + monacolin K (MK group), HFC + monascin (MS group), HFC + ankaflavin (AK group). The recommended dosage of RMR for an antiobesity effect is 2 g/day for humans in our previous study. [4] RMR included 9.82 mg/g monascin, 1.425 mg/g ankaflavin, and 2.89 mg/g monacolin K. The dosages of monascin, ankaflavin, and monacolin K were based on their concentrations in RMR. The adult dose of monascin (19.64 mg) in the MS group was equal to the monascin content of 2 g of RMR. The adult dose of ankaflavin (2.85 mg) in the AK group was equal to the ankaflavin content of 2 g of RMR. The adult dose of monacolin K (5.78 mg) in the MK group was equal to the monacolin K content of 2 g of RMR. The doses of the test substances used in this study were calculated according to Boyd's formula for body surface area for adult humans (weight: 65 kg; height: 170 cm). The rats in the MS, AK, and MK groups were orally administrated 0.55 mg/day monascin, 0.08 mg/day ankaflavin, and 0.16 mg/day monacolin K for 8 weeks, respectively.

Food consumption and body weight were recorded weekly. Feces were collected from rats on three consecutive days and oven-dried (65 °C) to a constant weight for the determination of fat content. At the end of the study, the rats were deprived of food for 16 h before being scarified by CO_2 asphyxiation. Blood samples were collected from the posterior vena cava and centrifuged at 700*g* for 10 min; the serum was stored at −20 °C until analyzed. Perirenal and epididymal fat pads were removed and weighed. Portions of the adipose tissue were immersed in 10% formaldehyde for histological inspection; other portions were frozen immediately in liquid nitrogen and stored at −80 °C for analysis of lipolysis and HR-LPL activity. Liver was excised and stored at −20 °C for the measurement of lipids. The experiment was reviewed and approved by the Animal Care and Research Ethics Committee of the National Taitung University.

Biochemical Analyses. The serum ketone body (hydroxybutyrate) and creatine phosphokinase (CPK) activity were measured using commercial kits (Randox Laboratories Ltd., Antrim, U.K.).

Lipolysis Assay. Adipose explants (0.1 g) of perirenal and epididymal fat pads from experimental rats were incubated in 1 mL of Krebs Ringer bicarbonate (KRB) buffer (20 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4{\cdot}7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 2% BSA; pH 7.4) at 37 °C for 1 h.[10] Glycerol was determined enzymatically from the supernatant by using a Randox kit.

HR-LPL Activity Assay. Adipose explants (0.1 g) of perirenal and epididymal fat pads was placed in 1 mL of KRB buffer supplemented with 10 U/mL heparin at 37 °C for 1 h. LPL activity was measured on the basis of its esterase property using *p*-nitrophenyl butyrate as a substrate. The TG hydrolase activity of LPL with synthetic TG substrates is inhibited by molar sodium chloride, and this property has been used to distinguish LPL activity from the activities of other lipases in plasma. Thus, HR-LPL activity was calculated from the productivity of *p*-nitrophenol using the following equation.[11]

$$C\ (\mu\mathrm{M}) = (A_{400}(0.15\ \mathrm{M\ NaCl}) - A_{400}(1\ \mathrm{M\ NaCl}))/0.012$$

where A_{400} (0.15 M NaCl) and A_{400} (1 M NaCl) are the absorbances of released *p*-nitrophenol at 400 nm in 0.15 M and in 1 M NaCl assay buffer, respectively, and 0.012 is the micromolar extinction coefficient of *p*-nitrophenol.

Adipose Tissue Histology. The adipose tissue samples were fixed in formaldehyde, embedded in paraffin, cut into 5 mm sections, and stained with hematoxylin and eosin. Cross-sectional areas of the adipocytes were calculated from the histogram according to Chen and Farese.[12] For the estimation of fat pad cell number, the lipid content of 0.3 g of fat tissue was extracted by using the method of Folch et al.[13] The total cell number in the fat pads was calculated by dividing the lipid content of the fat pad by the mean weight of cell lipids. The lipid weight of the average fat cell was calculated from the mean cell volume, assuming a lipid density of 0.915 (density of triolein).

Immunoblotting. Protein concentration was determined by the bicinchoninic acid (BCA) method. A total of 40 μg of total protein from each sample was applied as Western blot representative of three independent experiments according to the previous studies.[14,15] The samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking in a gelatin-NET solution, blots were incubated with monoclonal C/EBPα antibody (1:5000), monoclonal C/EBPβ antibody (1:2000), polyclonal PPARγ antibody (1:1000), monoclonal MTP antibody (1:200), and polyclonal NPC1L1 antibody (1:500) at room temperature for 1 h. Then, bands were incubated with specific horse radish peroxidase (HRP)-conjugated secondary antibodies (1:100 000) at room temperature for 1 h and visualized by enhanced chemiluminescence (ECL) substrate with a UVP AutoChemi Image system (UVP Inc., Upland, CA, USA). Protein loading was evaluated by anti-actin antibody (1:3000). Band intensities were quantified using UVP LabWork 4.5 software (UVP Inc.).

Statistics. Data are expressed as means ± standard deviation. Analysis of variance by Duncan's test and Pearson's product-moment correlation coefficient test was performed using SPSS version 10.0 software (SPSS Institute, Inc., Chicago, IL, USA). Differences with $p < 0.05$ were considered statistically significant.

Table 1. Effect of MK, MS, and AK on Body Weight, Food Intake, and Feed Efficiency in Male SD Rats Fed a High-Fat and High-Cholesterol Diet[a]

	weight gain (g)	food intake (g/8 weeks)	calorie intake (kcal/8 weeks)	feed efficiency (%)
NOR	116.3 ± 12.5 b	1724.1 ± 37.7 d	5758.6 ± 126.1 c	7.1 ± 0.4 a
HFC	154.1 ± 34.5 c	1281.9 ± 83.4 b	5340.3 ± 347.3 b	11.1 ± 1.8 c
MK	142.8 ± 17.0 c	1508.0 ± 97.5 c	6282.3 ± 406.1 d	9.3 ± 0.9 b
MS	75.3 ± 20.9 a	1098.1 ± 72.0 a	4574.8 ± 300.0 a	6.3 ± 1.2 a
AK	76.9 ± 18.3 a	1039.4 ± 68.6 a	4330.0 ± 285.6 a	6.7 ± 1.6 a

[a]Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$) ($n = 8$).

Table 2. Effect of MK, MS, and AK on Total Fat Pad, Perirenal Fat Pad, and Epididymal Fat Pad Weight and Body Fat Pad Percentage in Male SD Rats Fed a High-Fat and High-Cholesterol Diet[a]

	total fat		perirenal fat pads		epididymal fat pads	
	weight (g)	body fat ratio (%)	weight (g)	body fat ratio (%)	weight (g)	body fat ratio (%)
NOR	13.7 ± 3.3 a	2.6 ± 0.5 a	7.5 ± 1.8 a	1.4 ± 0.3 a	6.4 ± 1.5 a	1.2 ± 0.3 a
HFC	25.7 ± 5.5 c	4.7 ± 0.8 b	14.9 ± 3.0 c	2.7 ± 0.5 c	11.0 ± 2.5 b	2.0 ± 0.4 b
MK	17.6 ± 2.3 b	3.1 ± 0.4 a	10.9 ± 1.9 b	1.9 ± 0.3 b	6.5 ± 0.3 a	1.2 ± 0.1 a
MS	13.1 ± 2.4 a	2.7 ± 0.5 a	7.1 ± 1.5 a	1.5 ± 0.3 a	5.8 ± 0.9 a	1.2 ± 0.2 a
AK	14.7 ± 3.6 a,b	3.0 ± 0.7 a	8.4 ± 2.5 a	1.7 ± 0.5 a,b	6.3 ± 1.5 a	1.3 ± 0.2 a

[a]Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$) ($n = 8$).

Table 3. Effect of MK, MS, and AK on Cell Cross-Sectional Area and Number of Adipocyte Cells in Male SD Rats Fed a High-Fat and High-Cholesterol Diet[a]

	perirenal fat pads		epididymal fat pads	
	cell cross-sectional area (μm^2)	cell number × 10^4	cell cross-sectional area (μm^2)	cell number × 10^4
NOR	16 166 ± 2076 b	7.48 ± 0.97 ab	14 461 ± 3237 a	7.61 ± 1.25 b
HFC	23 183 ± 2286 c	16.79 ± 2.66 d	23 043 ± 941 c	13.89 ± 1.46 c
MK	14 572 ± 3350 a,b	6.28 ± 2.87 a	18 737 ± 2393 b	2.22 ± 0.44 a
MS	14 265 ± 2014 a,b	9.42 ± 1.56 b,c	14 969 ± 2032 a	6.94 ± 1.20 b
AK	13 372 ± 1929 a	9.86 ± 1.38 c	15 647 ± 2635 a	6.96 ± 1.42 b

[a]Two groups of the SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$) ($n = 8$).

RESULTS

Effects of Test Substances on Body Weight Changes, Food Consumption, and Food Efficiency. The animal experiment commenced under the condition that initial average body weight did not differ significantly ($p > 0.05$). Sacrifice and analysis were conducted 8 weeks after the experiment. Table 1 shows the weight gain of the HFC group was significantly higher than the NOR group ($p < 0.05$), whereas the weight gain of the MS and AK groups decreased significantly when compared to that of the HFC group (51.1% and 50.1%, respectively, $p < 0.05$) and was also significantly lower than the weight gain of the NOR group (35.3% and 33.9%, respectively, $p < 0.05$). The weight gain of the MK group decreased 7.3%, with no significant difference when compared to the HFC group ($p > 0.05$).

The food intake of the HFC group was significantly lower than the NOR group ($p < 0.05$). This may be because the unit calorie level of the high fat diet is higher (HFC: 4.17 kcal/g and NOR: 3.34 kcal/g). The food intake for the MS and AK groups was significantly reduced when compared to that for the HFC group, decreasing by 14.3% and 18.9%, respectively, $p < 0.05$). Conversely, the food intake of the MK group was increased significantly, by 17.6%, when compared to the HFC group ($p < 0.05$). The animal experiments of previous studies also observed that lovastatin suppresses appetite and may even be a factor for reducing body fat formation.[16] However, further research must be performed on the related mechanisms.

Feed efficiency [(body weight change/total food consumption) × 100%] can be considered as the efficiency for the same food intake to increase the body weight of an animal. Feeding high-fat diets significantly increased their feed efficiency in the HFC group ($p < 0.05$), potentially caused by the higher unit calorie level of the high-fat diet. The feed efficiencies of the MS, AK, and MK groups were all significantly lower than the HFC group, by 16.2%, 43.2%, and 39.6%, respectively ($p < 0.05$). For this reason, we speculate that monascin and ankaflavin, besides suppressing appetite and lowering food consumption, can also regulate and reduce body fat. Although the results from this

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study show monacolin K significantly increased food consumption and failed to suppress appetite, there was a reduction in feed efficiency.

The effects of the test substances on body fat pad weight are shown in Table 2. Total body fat pad, perirenal fat pad, and epididymal fat pad weight and body fat percentage in the HFC group all showed significant increases compared to the NOR group ($p < 0.05$). Consumption of test substances monacolin K, monascin, or ankaflavin all resulted in lower total body fat, perirenal fat pad, and epididymal fat pad weight and body fat percentage ($p < 0.05$), with monascin producing the best results. In the body fat reduction results, monascin and ankaflavin generally performed more effectively than monacolin K.

The effects of the test substances on cross-sectional area and number of adipocyte cells are shown in Table 3. The cross-sectional area and cell numbers of perirenal fat pads and epididymal fat pads of the HFC group were significantly increased by feeding a high-fat diet ($p < 0.05$ versus NOR group). The MK, MS, and AK groups all had significantly reduced adipocyte cross-sectional areas and cell numbers. The cross-sectional area reduction effect of ankaflavin was significantly higher than for monacolin K and monascin ($p < 0.05$), whereas the cell number reduction of monacolin K was significantly higher than that of monascin and ankaflavin ($p < 0.05$). These results show that monacolin K, monascin, and ankaflavin were able to inhibit adipocyte increase and cell volume expansion.

Monascin and ankaflavin were proven to repress adipose tissue C/EBPβ expression during preadipocyte differentiation in our previous study.[6] C/EBPβ is a key transcription factor and is positively correlated with preadipocyte differentiation.[17] The effects of monascin and ankaflavin consumption on adipose C/EBPβ protein expression are shown in Figure 1. The C/EBPβ protein expression was substantially higher in the HFC group than in the NOR group. A comparison of the MK and HFC groups shows no difference between the amounts of C/EBPβ protein expression, whereas the MS and AK groups respectively have 36.4% and 48.3% less C/EBPβ protein expression than the HFC group. Therefore, monascin and ankaflavin but not monacolin K inhibit preadipocyte differentiation by inhibiting C/EBPβ expression.

C/EBPβ expression induces PPARγ expression and further activates C/EBPα expression and therefore causes preadipocytes to differentiate into mature adipocytes.[17] The effects of monascin and ankaflavin consumption on adipose PPARγ protein expression are shown in Figure 1. Compared to the NOR group, PPARγ protein expression of the HFC group is substantially higher. Compared to the HFC group, the MK, MS, and AK groups all show expression reduced by 47.4%, 55.6%, and 64.5%, respectively. The expression of the MS and AK groups falls below even the NOR group by 15.9% and 32.9%, respectively. Therefore, monascin and ankaflavin inhibit differentiation by inhibiting C/EBPβ expression, thereby lowering PPARγ expression at relative amounts, whereas monacolin K begins inhibiting differentiation only at the PPARγ expression differentiation stage.

The expression of another transcription factor, C/EBPα, in adipose was significantly increased by the high-fat diet. However, compared to the HFC group, the MK, MS, and AK groups all show expression reduced by 22.7%, 25.2%, and 33.2%, respectively. Therefore, the later differentiation stage

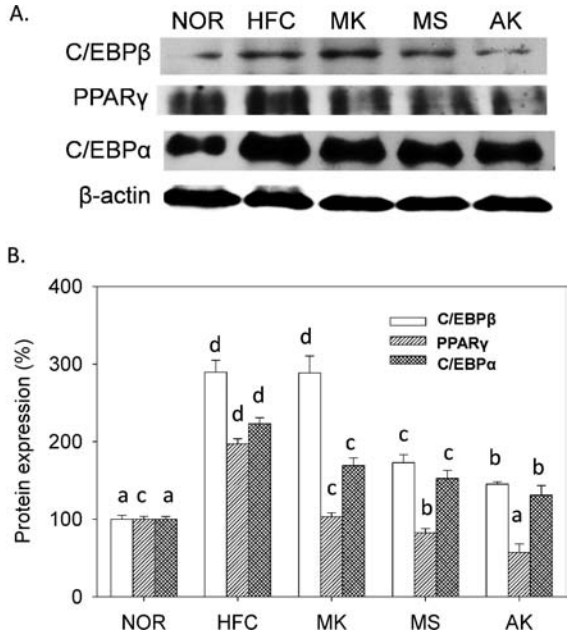

Figure 1. Effect of MK, MS, and AK on C/EBPβ, PPARγ, and C/EBPα protein expressions in adipose of male SD rats fed a high-fat and high-cholesterol diet. Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$).

was blocked by monascin, ankaflavin, and monacolin K due to the inhibition of C/EBPα expression.

Lipase in adipocytes mediates TG breakdown, thus elevating lipase activity in adipose tissue and impeding lipogenesis of adipocytes.[18] Table 4 shows that the HFC group had a

Table 4. Effect of MK, MS, and AK on Lipase Activity and HR-LPL Activity of Fat Pads in Male SD Rats Fed a High-Fat and High-Cholesterol Diet[a]

	lipase activity (U/g fat pad)	HR-LPL activity (U/g fat pad)
NOR	2.673 ± 0.267 a	0.243 ± 0.047 a
HFC	3.724 ± 0.355 b	0.423 ± 0.055 b
MK	2.995 ± 0.234 a	0.301 ± 0.078 a
MS	4.245 ± 0.322 c	0.276 ± 0.097 a
AK	4.164 ± 0.499 c	0.294 ± 0.072 a

[a]Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$) ($n = 8$).

significant increase in lipase activity when compared with the NOR group ($p < 0.05$). This may be related to the high-fat diet of the obese rats in the HFC group, causing an increase in lipids that consequently results in lipase function, causing lipolysis. Compared to the HFC group, the lipase activities of the MS and AK groups showed significant elevation ($p < 0.05$), whereas

the lipase activity of the MK group was as low as that of the NOR group. Therefore, monascin and ankaflavin increased lipase activity and facilitated lipolysis.

Lipoprotein lipase (LPL) in adipose tissue is a key enzyme for adipogenesis.[19] High fat and refined carbohydrate diets elevate adipose tissue LPL activity and lower striated muscle LPL activity, causing lipids to tend toward adipose tissue storage and the development of obesity.[20] Table 4 shows that the HFC group in this study had significantly increased HR-LPL activity compared to the NOR group ($p < 0.05$), demonstrating that the HFC group experienced adipogenesis toward the development of obesity. The MK, MS, and AK groups all showed significant decreases in HR-LPL activity of 28.8%, 34.8%, and 30.5%, respectively, compared to the HFC group ($p < 0.05$). In addition, the activity for these groups was as low as the NOR group ($p > 0.05$). Therefore, monascin, ankaflavin, and monacolin K contribute to the effect of *Monascus*-fermented products on reversing high-fat-diet-raised HR-LPL activity, thereby reducing lipogenesis.

A diet consisting of too much lipids is a cause of obesity and cardiovascular disease. NPC1L1 is a key protein for promoting lipid absorption in the small intestine. When the lipid levels in the cell fall, NPC1L1 is transported to the plasma membrane, thereby supplying lipids to the cell to catalyze endocytosis of NPC1L1 together with lipids in the cell.[21] The effects of MK, MS, and AK consumption on small intestinal tissue NPC1L1 expression are shown in Figure 2. The NPC1L1 expression is 163% greater in the HFC group than the NOR group. A high-cholesterol and high-fat diet potentially causes increased steroid lipid absorption. NPC1L1 protein expression in the MK, MS, and AK groups is reduced by 33.7%, 73.6%, and 26.1%, respectively, as compared to HFC group, showing that monacolin K, monascin, and ankaflavin reduce steroid lipid absorption. Furthermore, monascin reduced cholesterol absorption the most among the three test substances (2−3 times more than monacolin K and ankaflavin) and even 31% more than the NOR group. Thus, monascin may be an effective component for reducing steroid lipid absorption.

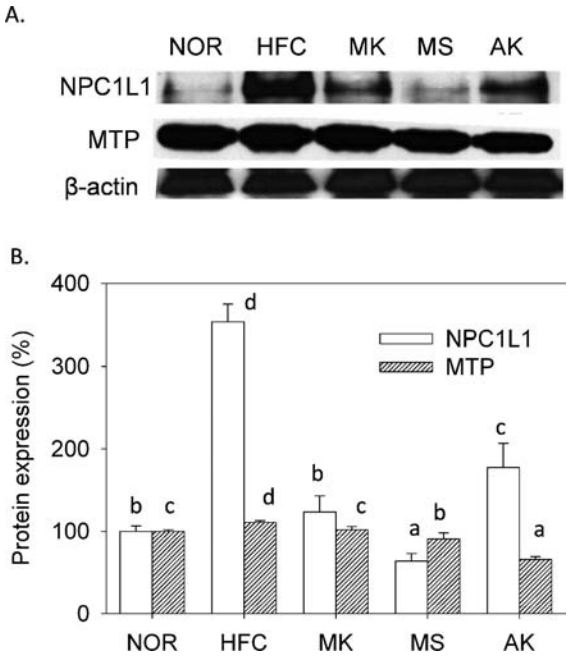

Figure 2. Effect of MK, MS, and AK on NPC1L1 and MTP protein expression in the small intestine of male SD rats fed a high-fat and high-cholesterol diet. Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$).

MTP protein, a downstream transporter protein of NPC1L1, carries TG, cholesterol ester, and phospholipid to apo B-48 and toward LDL-cholesterol assembly in small intestinal tissue.[22] As shown in Figure 2, a high-fat diet induced the expression of MTP protein in the HFC group. However, the increased trend is significantly decreased by the feeding of monascin and ankaflavin, and the lowering effect is stronger than that of monacolin K. Therefore, this result suggests that monascin and ankaflavin may not only repress lipid absorption but also prevent LDL assembly by the inhibition of NPC1L1 and MTP expression in small intestinal tissue.

A surplus of ketone body concentration in the blood (a safety indicator) is caused by abnormal glucose metabolism, resulting in accelerated lipolysis and overproduction of acetyl-CoA. With no means for the acetyl-CoA to sufficiently enter the tricarboxylic acid (TCA) cycle, it transforms more ketone bodies and exacerbates the problem. The acidic ketone bodies cannot be expelled appropriately from the body. Consequently, they accumulate in the blood and easily develop into acidosis, called ketoacidosis.[23] The objective of this study is to determine whether the test substances cause ketone body overproduction. The results are shown in Table 5. The ketone body concentrations of the MK, MS, and AK groups were significantly lower than for the NOR group (normal food intake group; $p < 0.05$), indicating that the test substances monacolin K, monascin, and ankaflavin did not cause ketone body overproduction as a consequence of reduced body fat. We speculate that the reason for ketone body levels of the three test substance groups being significantly lower than the NOR group is because of a reduction in body fat formation and not increased lipolysis. When fat production amounts were reduced, fat production in the body decreased, and it prevented ketone body biosynthesis.

Table 5. Effect of MK, MS, and AK on Serum Ketone Body Concentration and Creatine Phosphokinase Activity in Male SD Rats Fed a High-Fat and High-Cholesterol Diet[a]

	ketone bodies (mmol/L)	CPK activity (U/L)
NOR	1.73 ± 0.36 b	42.6 ± 16.7 a
HFC	1.74 ± 0.23 b	186.5 ± 46.9 b
MK	0.28 ± 0.14 a	180.9 ± 40.8 b
MS	0.26 ± 0.16 a	61.5 ± 35.2 a
AK	0.34 ± 0.15 a	76.4 ± 27.6 a

[a]Two groups of SD rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic and obese SD rats were administrated monacolin K (0.16 mg/day 400 g bw) (MK group), monascin (0.55 mg/day 400 g bw) (MS group), and ankaflavin (0.08 mg/day 400 g bw) (AK group). Mean values within each column with different superscripts are significantly different ($p < 0.05$) ($n = 8$).

The cholesterol-lowering agent statins (monacolin K) may cause muscle damage and rhabdomyolysis;[24] however, CPK activity is an important indicator for the evaluation of muscle damage. Overactive CPK in the blood indicates that the heart, brain, or muscle tissue is damaged, thereby allowing CPK to leak into the bloodstream.[25] The results in Table 5 show no significant difference in CPK activity among the NOR, MS, and AK groups ($p > 0.05$). This implies that consuming the test substances monascin and ankaflavin does not result in muscle tissue damage. However, consumption of a high-fat diet in the HFC group or monacolin K in the MK group both showed relatively higher levels of CPK activity ($p < 0.05$).

DISCUSSION

Results from previous animal experiment research observed that adding 0.4% or 2% (w/w) RMR to a high fat diet significantly inhibits kidney and epididymal adipocyte enlargement, mitigates adipose tissue accumulation, and slows weight gain.[4] Kim et al. noted that daily consumption of 0.1 or 0.2 mg of *Monascus*-fermented pigment derivatives L-tryptophan or L-leucine ethyl ester benefits the antiobesity and hypolipidemic effect.[26] Our previous cell tests also showed that monascin and ankaflavin are potentially effective agents for reducing body fat.[6]

This study is the first to investigate the *in vivo* effects of *Monascus*-fermented secondary metabolites (monascin and ankaflavin) on the antiobesity effect and the regulation of differentiation and lipogenesis in adipose and lipid absorption in the small intestine. The results show that both monascin and ankaflavin significantly reduce body weight gain, food intake, feed efficiency, fat mass or weight, body fat percentage, adipocyte cross-sectional area, and adipocyte cell number in rats ($p < 0.05$), as well as significantly increase lipase activity to enhance lipolysis efficiency and significantly lower HR-LPL activity to prevent lipogenesis and oil droplet accumulation. Chen et al. (2008) observed that RMR causes an antiobesity effect via increasing lipolysis efficiency and HR-LPL activity in adipose tissue to reduce adipogenesis.[4] From the finding that the body fat formation reduction factors for RMR show similar trends to monascin and ankaflavin, we speculate that monascin and ankaflavin are effective components of *Monascus*-fermented RMR in reducing body fat formation.

Preadipocyte differentiation into mature adipocytes is a crucial key to the adipose formation process. C/EBPβ is the early transcription factor to be expressed, inducing PPARγ expression. PPARγ must form heterodimers with another transcription factor, retinoid X receptor (RXR). After binding with a ligand, it binds to DNA and engages in transcriptional activity. Transcription factor adipocyte determination and differentiation factor 1 (ADD1) produces endogenous ligands and induces PPARγ expression. Activated PPARγ induces C/EBPα expression. Cells officially enter the differentiation phase and begin to manifest the property characteristics of mature adipocytes. Inspection of the cell morphology shows the cell slowly changing from its original fibrous form into a round shape, even forming oil droplets within the cell.[27] PPARγ and C/EBPα possess positive feedback functions and reinforce the expression of each other. For this reason, once differentiation has commenced, these decisively instrumental transcription factors continue to be expressed.[17] Jeon et al. used RMR extract (RE) treatments on 3T3-L1 preadipocytes and observed the effects of RE on preadipocyte differentiation.[28] RE treatment (2 mg/mL) significantly inhibits the gene expression of C/EBPα and PPARγ and concurrently decreases PPARγ protein expression. RE also significantly reduces the gene expression of aP2 and leptin by approximately 52% and 51%, respectively. Consequently, the inhibitory effect of RE on adipocyte differentiation might be activated through mediating the expression of relevant transcription factors and other specific genes.[28] Our previous cell research further showed that monascin and ankaflavin effectively suppress C/EBPβ, C/EBPδ, PPARγ, and C/EBPα mRNA and protein expression, as well as lower TG accumulation.[6] The results in Figure 1 can be used to deduce potential reasons for the reduction in body fat. Monascin and ankaflavin decrease C/EBPβ expression during early preadipocyte differentiation, as well as decrease PPARγ expression during mid-differentiation and C/EBPα expression during late differentiation (Figure 1). This study is the first to confirm that the consumption of monascin and ankaflavin by obese rat models is able to inhibit the expression of key transcription factors in the adipocyte differentiation process.

Mature adipocyte size is also affected by lipase and LPL activity. Lipase and LPL activity are adipocyte lipolysis and adipogenesis indicators, respectively. Increasing adipose tissue lipase activity is able to reduce body fat gain through the lipolysis of droplets.[18] Diets high in fat and refined carbohydrates increase adipose tissue LPL activity and decrease striated muscle LPL activity, causing lipogenesis and obesity.[20] Previous results from 3T3-L1 cell tests showed that monascin and ankaflavin significantly stimulated lipolysis and decreased HR-LPL activity in mature adipocytes.[6] The *in vivo* results of this study show that monascin and ankaflavin also significantly increase lipase activity (14% and 10.7%, respectively, $p < 0.05$) and decrease HR-LPL activity (34.8% and 30.5%, respectively, $p < 0.05$), thereby down-regulating lipogenesis and reducing body weight and body fat or lipid weight (Table 4). These results imply that monascin and ankaflavin are more dependent on the effects of decreasing lipogenesis than on promoting lipolysis. LPL expression is mediated by the activation of PPARγ by cognate ligands, as LPL is a downstream gene of PPARγ. The PPARγ/RXR complex binds to the PPRE present in the promoter region of the LPL gene and increases LPL gene expression.[29] The induction of lipoprotein lipase synthesis by PPARγ is mainly in the mature adipocytes in order to increase local generation of free fatty acids.[30,31] However, both monascin and ankaflavin are proven to inhibit PPARγ expression in this study. This could be one of the primary factors of monascin- and ankaflavin-mediated inhibition of lipogenesis.

Monascin and ankaflavin have been proven as hypolipidemic agents in previous research,[8] but the mechanisms are not clear. This study is the first to observe that monascin significantly inhibits NPC1L1 protein expression, causing the reduction of the absorption of exogenous lipids. NPC1L1 is a type of integral membrane protein that exists on the brush border membrane of the jejunum. It absorbs cholesterol vesicle endocytosis and has a critical function in the absorption of cholesterol.[32] However, the NPC1L1 inhibitor treatment is proven to reduce plasma triglyceride levels in some animal models fed diets high in fat and cholesterol, suggesting that NPC1L1 expression may modulate fat metabolism.[33] In addition, less fatty acid transport protein 4 (FATP4) expression is proven in intestinal scrapings of NPC1L1(−/−)- and ezetimibe-treated mice, suggesting NPC1L1 expression mediated the intestinal absorption of long-chain fatty acids.[34] The previous study also indicates that the NPC1L1 inhibitor ezetimibe improves metabolic syndrome and nonalcoholic

fatty liver disease in obese rats via the inhibition of lipid absorption. According to the above-mentioned data, suppressing NPC1L1 expression is able to reduce lipid absorption and further reduce absorption of calorie intake and cardiovascular risk factors simultaneously. However, although monascin and ankaflavin are both yellow pigments, monascin exhibits more significant inhibition of NPC1L1 protein expression than ankaflavin.

Monacolin K is a statin compound and previously regarded as a hypolipidemic component of *Monascus*-fermented products. Previous research also confirms its antiobesity effects.[4] In this study, some indicators show that it possesses antiobesity effects. However, there are also many indicators that show that the effects of monacolin K are significantly lower than those of monascin and ankaflavin, such as for reductions in weight gain, feed efficiency, and perirenal fat pad weight. Monacolin K was the default to lower body weight gain, which may have resulted from higher food intake and calorie intake in the MK group (Table 1). Increased appetite stimulation by monacolin K is unclear currently. However, the higher calorie intake did not result in an increased fat pad ratio. Therefore, the monacolin K-mediated down-regulation on obesity factors still caused a fat-lowering effect.

Nevertheless, with regard to side-effect testing, overactive CPK in the blood indicates that the heart, brain, or muscle tissue is damaged, allowing CPK to leak into the bloodstream.[25] The results in Table 5 show that the CPK activity in the NOR, MS, and AK groups showed no significant difference ($p > 0.05$), implying that monascin and ankaflavin do not result in muscle tissue damage. However, the CPK activity is significantly greater in the HFC and MK groups than in the other three ($p < 0.05$). A high-fat and high-cholesterol diet could easily cause heart disease and heart damage. However, this study shows that monacolin K effectively prevent high-fat-diet-induced cardiovascular disease. Therefore, the reason for increased CPK activity in the MK group is unrelated to a concurrent phenomenon caused by cardiovascular disease; however it is potentially related to the independent effects of monacolin K. The side-effect of myopathy caused by monacolin K reported in previous studies may deteriorate from mild muscle weakness into severe and fatal rhabdomyolysis.[24,35] Monacolin K-induced CoQ10 deficiency may harm muscle energy metabolism and exacerbate myopathy.[36] Muscle damage releases CPK into the bloodstream, causing an increase in CPK blood concentration that is accompanied by muscle weakness or pain, potentially devolving into rhabdomyolysis.[25] Furthermore, regarding the safety of monascin and ankaflavin, they are common and natural yellow pigments of *Monascus* used in food processing. In our previous study, monascin and ankaflavin were used to obtain a hypolipidemic effect in a hyperlipidemic animal model. A 5-fold dosage of the two compounds was still harmless in the liver and kidney according to histochemical staining.[8] Furthermore, a *Monascus*-fermented product was also proven to be a safe food via 28-day and 90-day toxicity evaluation tests in the previous study.[37,38]

The results of this study show that monascin and ankaflavin were able to significantly decrease body weight gain, feed intake, food efficiency, fat mass or weight, body fat percentage, adipocyte cross-sectional area, and adipocyte cell number in rats. These results may be a result of the inhibitory effects of monascin and ankaflavin on the regulation of differentiation, in which monascin and ankaflavin inhibited C/EBPβ protein expression in the early stages and further decreased PPARγ expression and C/EBPα protein expression, all inhibiting adipocyte differentiation. Regarding lipogenesis regulation in mature adipocytes, monascin and ankaflavin increased lipase activity and decreased HR-LPL activity, thereby reducing lipogenesis. Furthermore, monascin had a significant effect on suppressing NPC1L1 protein expression associated with small intestine tissue cholesterol and lipid absorption. These antiobesity effects of monascin and ankaflavin would not result in increased CPK activity and ketone body levels.

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Notes

The authors declare no competing financial interest.

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