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Anti-lipogenic Effects of Tannic Acid in 3T3-L1 Adipocytes and in High Fat Diet-fed Rats

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Abstract Anti-lipogenic effects of tannic acid on 3T3-L1 adipocytes as well as on rats fed high fat diet (HFD) were investigated. Tannic acid stimulated lipolysis through suppression of the leptin secretion and an increase of glycerol release in a dose dependent manner in 3T3-L1 adipocytes. For animal study, the rats were fed either HFD or HFD supplemented with 1%(w/w) tannic acid (HFDT) for 12 weeks, respectively. Body weight gain, liver weight, and visceral fat mass in rats fed HFDT were significantly decreased compared to those of rats fed HFD. The lipid profiles of HFDT group were significantly decreased compared with HFD group in the serum and liver, whereas fecal total cholesterol excretion was increased in HFDT group. These results suggest that anti-lipogenic effect of tannic acid in 3T3-L1 adipocytes and in rats fed HFD may be due to the stimulation of lipolysis and the reduction of lipid levels.

Keywords: tannic acid, 3T3-L1 adipocyte, high fat diet, fecal fat excretion, anti-lipogenic effect

Introduction

A growing body of literature suggests that high fat diet is directly linked to development of obesity, which leads to diabetes, dyslipidemia, cardiovascular disease (CVD), cancer, and sleep-breathing disorder (1). Diets with high saturated fat promote obesity in rodents and humans whereas diets with high polyphenols appear to prevent or attenuate the development of these diseases (2-9).

Obesity, especially abdominal obesity, has an association with dyslipidemia characterized by increasing triglyceride (TG) and decreasing high-density lipoprotein cholesterol (HDL-C) concentration. Also, reduced lipolytic activity may contribute to accumulation of TG in adipose tissue and thus development of obesity (10-13). The adipocyte hormone, leptin, has a central role in the regulation of food intake, energy expenditure, and body fat stores (14). Leptin levels are increased in obesity, and leptin exhibits cardiovascular actions that may contribute to increased cardiovascular risk (15,16). Increased leptin secretion and decreased catecholamin-induced lipolysis was observered in obesity, and increase of free fatty acids (FFAs) by lipolysis has been reported to inhibit leptin expression and secretion in adipocytes (17,18).

Tannins are the most abundant flavonol-type flavonoid in the human diet (2). Based on their chemical structures, tannins are further categorized into hydrolysable and condensed tannins. The main components of hydrolysable tannins are tannic acid (19). Tannic acid has a multiple biological activities, including anticarcinogenic (20), antimutagenic (21), antidiabetic (7), antioxidative (22,23), and hypocholesterolemic effects (9). Although condensed tannin has many report about antiadipogenesis and lipolysis in 3T3-L1 adipocytes (24,25), hydrolysable tannin (tannic acid) has not been studied systematically for its potential functions in the processes of adipogenesis in 3T3-L1 adipocytes and in high far diet (HFD)-fed rats. Therefore, the objective of the present study was to determine the direct effects of tannic acid on leptin secretion and glycerol release in 3T3-L1 adipocytes and whether its antilipogenic effects were related to alterations on lipid profiles in HFD-fed rats.

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Materials and Methods

Materials Tannic acid was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were used ACS grade.

Cell culture and differentiation 3T3-L1 preadipocytes (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin, at 37°C in a humidified 5% CO_2. Briefly, 2 days after reaching confluence, the cells were treated with 0.05% trypsin/ethylendiamine tetraacetic acid (EDTA) and were collected with centrifugation at 800×g for 5 min. Differentiation to the adipocyte form was induced by incubating 3.3×10^3 cells in DMEM containing 5 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM isobutyl-methylxanthine for 3 day in 12 well culture plates. After 48 hr, medium was changed to DMEM with 5 μg/mL insulin and subsequent medium changes occurred every second day. Tannic acid was dissolved in DMEM with 0.1% concentration, and then filtered with 0.2 μm filter, and added to the fully differentiated cell.

Quantification of leptin Quantification of leptin released to the medium was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, St. Charles, MO, USA). Rat anti-mouse leptin IgG 2 μg/mL was added in Maxisorb ELISA

plate (Nunc-immunosorb, Maxisorb, Denmark) and incubated for overnight. The plate was washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and tannic acid (0.1%) 100 μL was added to the medium and followed by incubation for 1 hr. The biotinylated rabbit anti-mouse leptin IgG 200 μg/mL was added to the mixture after it was washed with PBS-T 3 times. Then the mixture was incubated for 1 hr and then washed 3 times with PBS-T. The extravidinn-horse radish peroxidase was added to the mixture and then mixture was incubated for 1 hr and washed 3 times with PBS-T. Immuno-reactivity was indicated after reaction for 30 min by the addition of 100 μL tetramethylbenzidine dihydrochloride substrate. The reaction was terminated by adding 2 M H_2SO_4 50 μL. The intensity of color was measured at 450 nm. Recombinant mouse leptin was used as a standard.

Lipolysis Glycerol release into the media at 96 hr was assayed to evaluate lipolysis. Glycerol was measured spectrophotometrically using Free Glycerol reagent (Sigma-Aldrich), according to the methods described in the manufacturer's manual. Tannic acid (0.1%) was added 10 μL and pre-warmed free glycerol reagent 1 mL, and then incubated at 37°C for 5 min. Glycerol standards (0-25 μg) were run in parallel with test samples, and the concentrations of released glycerol were calculated based on the standard calibration curve.

Animals and diets The male Sprague-Dawley rats, 6 weeks old, were obtained from Bio Genomics Inc. (Seoul, Korea). Care of the animals and all experimental procedures were conducted in accordance with the 'Institutional Guidelines for Animal Research' of the Pusan National University. The rats were fed chow diet for 1 week and then divided into 2 groups of 10 rats each. Two rats were housed in 1 plastic cage and kept at 22±1°C and 55±5% relative humidity in a room under a 12-hr light-dark cycle. Diet was prepared according to the AIN-76 guideline and was prepared to be isocaloric between the 2 experimental groups as shown in Table 1 (460 kcal/100 g of diet). The HFD contained 20% fat (16% lard and 4% corn oil), which was equivalent to 31% of total energy. Rats were fed either HFD or HFD supplemented with 1% tannic acid (HFDT) for 12 weeks. The animals were given food and distilled water *ad libitum* during the experimental period. Food consumption and weight gain were measured daily and weekly, respectively. Feces were collected during the final 3 days and were used for determination of fecal lipids. At the end of the experimental period, the rats were anesthetized with ether after 12-hr fasting. Blood obtained from the heart was immediately centrifuged and plasma was stored at −70°C until use. Liver was perfused with PBS through the portal vein to expel the blood and then removed.

Table 1. Composition of experiment diets[1)]

Ingredients	Level (g/100 g of diet) in experimental group[2)]	
	HFD	HFDT
Casein	20	20
Sucrose	40	40
Corn starch	10	10
Corn oil	5	5
Lard	15	15
Cellulose	5	5
DL-Methionine	0.2	0.2
Mineral mixture*	3.5	3.5
Vitamin mixture**	1.0	1.0
Choline bitrate	0.3	0.3
Tannic acid	-	1
Total	100	101

[1)]The diet was prepared according to the AIN-76 guideline and was prepared to be isocaloric, 460 kcal/100 g, between experimental groups; *AIN-76 mineral mixture; **AIN-76 vitamin mixture.
[2)]HFD, high fat diet; HFDT, HFD supplemented with 1% tannic acid.

Serum lipids The concentrations of total cholesterol (TC), TG, and HDL-C in plasma were determined by enzymatic colorimetric methods using commercial kits (Asan Pharmaceutical, Seoul, Korea). Low-density lipoprotein-cholesterol (LDL-C) was accomplished according to the procedures described by Friedwald *et al.* (26). The atherogenic index (AI) was calculated as (TG − HDL-C)/(HDL-C).

Hepatic and fecal lipids The concentration of hepatic and fecal lipids was determined by the method of Folch *et al.* (27) and then determined with the same kits used for plasma TC and TG analysis.

Statistical analysis The results were presented as mean± SD. Statistical analysis was performed using the ANOVA for *in vitro* and Student's *t*-test for *in vivo* study (SPSS 12.0, SPSS Inc., Chicago, IL, USA) at the $p<0.05$ significance levels.

Results and Discussion

Effect of tannic acid on leptin secretion and lipolysis Tannic acid showed a concentration-dependent inhibition of leptin secretion in differentiated 3T3-L1 adipocytes (Fig. 1). The inhibitory effect of tannic acid on leptin secretion was significant at concentrations of 1 and 10 μg/mL (65 and 13% of control). The effects of tannic acid on lypolysis were evaluated by determining the amount of glycerol release into the media in differentiated 3T3-L1 adipocytes (Fig. 1). Tannic acid stimulated a concentration-dependent increase in glycerol release, which was significant at concentration of 1 and 10 μg/mL (128 and 140% of control). The increase in glycerol release had negative correlation ($r=-0.5592$, $p<0.05$) with inhibition of leptin secretion in tannic acid treated 3T3-L1 adipocytes.

The results of this study showed that tannic acid inhibited the leptin secretion and stimulated the glycerol release from 3T3-L1 adipocytes, as indicated by the stimulation of lipolysis. A continuous high fat diet may be result in increased serum leptin, whereas plant polyphenol suppressed lipogenesis by the inhibition of leptin secretion and the stimulation of lipolysis in rat adipocytes (28,29). Furthermore, tannic acid stimulates glucose transport and inhibits adipocyte differentiation in 3T3-L1 cells, suggesting that tannic acid must have altered not only the expression

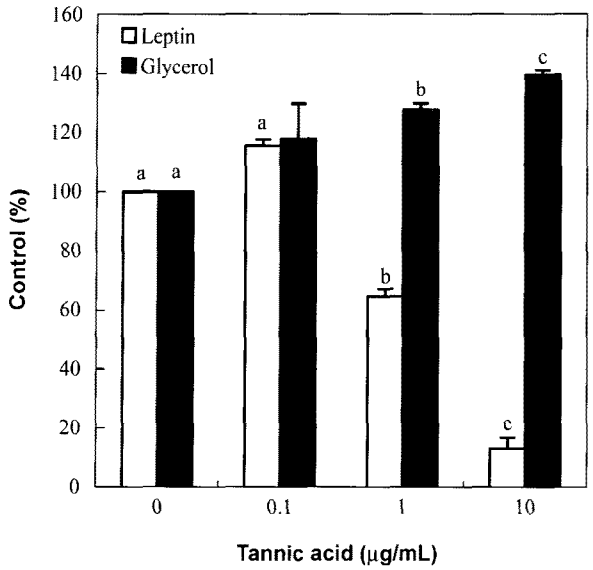

Fig. 1. Effects of tannic acid on glycerol release and leptin secretion in 3T3-L1 adipocytes over 96 hr in culture. Data are expressed as mean±SD. [a-c]Data are significantly different by ANOVA followed Duncan's multiple range test at the 0.05 level of significance.

of genes involved in the adipogenesis, but also other earlier genes involved in cell proliferation of preadipocytes (6,7). Therefore, the results of this study showed that the decrease of leptin secretion and increase of glycerol release by tannic acid in 3T3-L1 adipocytes may be attributable to the decrease of adipose tissue in HFD-fed rats. In the present study, an inverse relationship between lipolysis and leptin secretion was observed. It has been shown that FFAs can inhibit leptin expression and secretion in adipocytes (18,30).

Effect of tannic acid on body weight, food intakes, and food efficiency ratio (FER) Initial body weights of 2 groups were similar, however, after 12 weeks, body weight gain was significantly lower in the HFDT groups than in the HFD group. Food intakes were not affected by the experimental diet. FER was significantly higher in the HFD group than in the HFDT group, and the HFDT group exhibited a same FER ratio (Table 2). We demonstrated that daily consumption of tannic acid tended to suppress body weight gain by 7.3% without affecting food intake in HFD-fed rats. Leptin, an adipocyte-derived hormone, is a key factor in regulating food intake, body weight, energy expenditure, body fat stores, and insulin signaling (31). High levels of serum leptin are commonly associated with

Table 3. Liver and visceral fat weights of rats fed high fat diet containing tannic acid for 12 weeks[1)]

Group[2)]	Liver weight (g)	Total visceral fat (g)
HFD	16.75±1.09	32.84±6.09
HFDT	14.33±1.12*	26.09±6.21*

[1)]Values are mean±SD (n=10); *data are significantly different from HFD group at $p<0.05$ by Student's t-test.
[2)]HFD, high fat diet; HFDT, HFD supplemented with 1% tannic acid.

obesity and could represent a state of leptin resistance (32). On the other hand, polyphenol that inhibits leptin secretion, appears to stimulate glycerol release by increasing the lipolysis metabolism of TG to glycerol and fatty acid through an increase in the activity of hormone sensitive lipase (4,5). Leptin and stearoyl-CoA desaturase-1 gene expression was dose-dependently decreased by epigallocatechin gallate in white adipose tissue, which could be one reason for the reduced fat accumulation in diet-induced obesity model (33). Also, this result demonstrated that tannic acid decrease leptin secretion in adipocytes *in vitro*, suggesting that tannic acid therapy may be associated with altered leptin homeostasis contributing to weight gain and obesity *in vivo*.

Effect of tannic acid on liver and visceral fat weights As shown in Table 3, the liver weight was significantly lower in the HFDT group than in the HFD group. The supplementation of tannic acid significantly lowered the visceral fat weights by 21% compared to the HFD group. A naturally occurring peroxisome proliferators activated receptor γ (PPARγ) agonist that can regulates lipid and glucose metabolism through increasing of PPARγ and adipose triglyceride lipase in 3T3-L1 and obesity due to high fat diet or leptin-deficiency (34,35). Also, the fat accumulation in liver and the size of adipocytes from visceral fat decreased in rats fed biocellulose-containing diet (36).

Effect of tannic acid on plasma, hepatic, and fecal lipids The concentrations of plasma, hepatic, and fecal lipids are shown in Table 4. The supplementation of tannic acid significantly lowered plasma TG concentration by 24%, and TC concentrations by 11% compared to the HFD group, respectively. The HDL-C and ratio of HDL-C/TC were significantly increased by the supplementation of tannic acid compared to the HFD group. The hepatic TG concentrations also were significantly lower in the HFDT group by 24% than in the HFD group, and those for hepatic TC concentrations were 11%, respectively ($p<$

Table 2. Change in body weight, food intake, and food efficiency ratio (FER) of rats fed high fat diet containing tannic acid for 12 weeks[1)]

Group[2)]	Initial weight (g)	Final weight (g)	Weight gain (g/day)	Food intake (g/day)	FER[3)]
HFD	110.76±2.35	513.13±20.52	4.68±0.22	19.03±1.59	0.25±0.13
HFDT	110.73±4.34	480.00±37.13*	4.34±0.37*	19.21±1.33	0.22±0.29*

[1)]Values are mean±SD (n=10); *data are significantly different from HFD group at $p<0.05$ by Student's t-test.
[2)]HFD, high fat diet; HFDT, HFD supplemented with 1% tannic acid.
[3)]FER=weight gain (g/day)/food intakes (g/day).

Table 4. Effects of tannic acid on lipid profiles of plasma, liver, and feces of rats fed high fat diet for 12 weeks[1)]

Parameter	Group[2)]	
	HFD	HFDT
Serum		
TG (mg/dL)	204.28±11.73	155.96±18.75*
TC (mg/dL)	120.17±9.91	107.17±10.67*
HDL-C (mg/dL)	41.48±3.94	47.02±7.01*
LDL-C (mg/dL)	38.15±3.62	30.15±0.66
HDL-C/TC	0.34±0.04	0.44±0.08*
AI[3)]	1.95±0.40	1.28±0.39*
Liver		
TG (mg/g)	87.97±2.59	66.80±1.44*
TC (mg/g)	26.73±1.69	23.74±1.36*
Feces		
TG (mg/g)	25.31±3.59	26.14±0.00
TG (mg/g)	9.34±0.48	11.37±1.79*

[1)]Values are mean±SD (n=10); *data are significantly different from HFD group at $p<0.05$ by Student's *t*-test.
[2)]HFD, high fat diet; HFDT, HFD supplemented with 1% tannic acid.
[3)]Atherogenic index=(TC−HDL-C)/HDL-C.

0.05). Excretion of TG in the feces was not significant between the 2 groups. However, tannic acid supplementation significantly enhanced the excretion of fecal TC by 22% compared to the HFD group. Inhibition of digestion and absorption of dietary fat by flavonoid has been used as target in obesity treatment (37). In the present study, tannic acid reduced the weight gain, visceral fat mass as well as plasma and hepatic lipid levels partly via the increased fecal lipids in HFD-fed rats. This is in agreement with previous study which showed lipid lowering effect in rat after dietary supplementation with tannin (38). Yugarani *et al.* (9) reported that tannic acid (100 mg/rat/day) reduced the plasma TC, LDL-C, TG, and fat deposition in HFD-fed rats for 10 weeks. Although, tannic acid did not affect the plasma LDL-C in our study, the plasma TC and atherogenic indexes were lowered by tannic acid, while HDL-C and HDL-C/TC increased. This information suggests that tannic acid might have beneficial effects on CVD and obesity by decreasing the lipid levels and increasing the fecal lipid excretion. Furthermore, other flavonoids have been shown to inhibit cholesterol biosynthesis and hepatic lipogenesis in hepatic cells (39,40). In fecal lipid levels, tannic acid supplementation led to increase of cholesterol excretion. Numerous studies have reported the effect of tannic acid on enhancing the excretion of sterols in the feces (41). Tannic acid showed a significant effect on inhibiting the pancreatic lipase activity *in vitro* and increasing the fecal fat excretion (42). Thus, tannins have a pronounced anti-hypercholesterolemic effect by enhancing reverse cholesterol transport and also by reducing intestinal cholesterol absorption and increasing bile acid excretion (43). The precise mechanism of the effect of tannic acid on HFD condition is still unknown, and further investigation should be undertaken.

In conclusion, tannic acid suppressed the leptin secretion and increased the glycerol release in differentiated 3T3-L1 adipocytes. Also, anti-lipogenic effect of tannic acid in HFD-fed rats may be due to the decreased lipid levels through increasing of fecal fat excretion. These results suggest that tannic acid may have the potential to anti-lipogenic compounds in high fat condition.

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