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Safflomide Increases the Expression of Adiponectin *in Vitro* and *in Vivo*: Potential Implication for Hypoadiponectemia, Visceral Obesity, and Insulin Resistance

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ABSTRACT: Safflomide (*N*-caffeoyltryptamine) is a phenolic amide with serotonin receptor antagonist and antioxidant activities. We investigated the potential effects of safflomide on the expression of adipokines *in vitro* and *in vivo*. Safflomide did not affect the expressions of TNF-α, IL-6, and MCP-1/CCL2 in hypertrophic 3T3-L1 cells but upregulated adiponectin mRNA 1−5-fold at concentrations between 1 and 20 μM ($p < 0.05$). Because safflomide is a non-selective 5-HT receptor antagonist and because the expression of 5-HT2A receptor is often inversely correlated to adiponectin expression, the potential effects of 5-HT receptor antagonist activity of safflomide on the expression of adiponectin was further investigated in 3T3-L1 cells. At the concentration of 10 μM, safflomide was able to increase adiponectin protein production in 3T3-L1 cells more than 4-fold ($p < 0.05$), which was greater than the 5-HT2A antagonist ketanserin. The upregulation was partially suppressed by treatment with 5-HT2A agonists (serotonin and α-Me-5-HT), suggesting that safflomide may upregulate adiponectin expression more than by blocking 5-HT2A receptors in 3T3-L1 cells. Likely, the upregulation was also attributed to the antioxidant activity of safflomide because two safflomide analogues (*N*-cinnamoyltryptamine and *N*-coumaroyltryptamine) with less antioxidant activity were not as potent as safflomide. Rats supplemented with safflomide (3 mg/day) in a high-fat diet showed a significant plasma adiponectin increase (more than 30%) with a significant reduction in body weight, visceral fat, and improved insulin resistance compared to non-supplemented rats, demonstrating the *in vivo* activity of safflomide. These data suggest that safflomide may have beneficial effects on obesity-related conditions, such as low adiponectin, visceral obesity, and insulin resistance.

KEYWORDS: *Safflomide, antioxidant, adiponectin, serotonin receptor, obesity, insulin resistance*

■ INTRODUCTION

Adiponectin is an adipocyte-derived adipokine with antidiabetic, antiatherogenic, and anti-inflammatory functions.[1−5] Adiponectin is found in the blood in trimeric, hexameric, and high-molecular-mass species, which have been reported to play distinctive roles in the regulation of energy homeostasis.[6−9] In adipocytes, the expressions of adipokines (e.g., adiponectin and leptin) are modulated depending upon adipocyte hypertrophy (e.g., size and content). Also, the expressions of cytokines, such as TNF-α, IL-6, and CCL2/MCP-1, probably from adipocyte-infiltrated macrophages, are often varied according to the extent of adipocyte hypertrophy.[10−14] Adiponectin, in particular, is significantly decreased in hypertrophic fat cells. This decrease is frequently followed by increasing insulin resistance, which is one component of the metabolic syndrome strongly associated with low-grade inflammation, obesity, diabetes, cardiovascular disease, and hypertension.[15−18] Therefore, the upregulation of adiponectin is considered beneficial in alleviating obesity-related diseases, such as diabetes, cardiovascular disease, and hypertension.[19−21]

Safflomide is a phytochemical belonging to a group of serotonin-derived phenylpropenoid amides found as minor components in plants, such as *Carthamus tinctorius*, *Centaurea cyanus*, *Coffea* sp., and *Ipomoea obscura*.[22−25] Previous studies suggested that this amide has non-selective 5-HT receptor antagonist activity along with antioxidant and anti-inflammatory activities.[26,27] Interestingly, recent studies suggested that 5-HT receptors (e.g., 5-HT2A) are associated with regulating the expression of adiponectin in hypertrophic 3T3-L1 cells.[28,29] In fact, the upregulation of 5-HT2A receptor expression was reported to be positively correlated with the decreased expression of adiponectin, and 5-HT2A receptor antagonists were able to increase adiponectin expression in hypertrophic 3T3-L1 cells.[28,29] However, the potential effects of safflomide on the expression of adiponectin have not been investigated either *in vitro* or *in vivo*. Therefore, the potential effect of safflomide on the expression of adiponectin was investigated in hypertrophic 3T3-L1 cells, especially related to 5-HT2A receptor antagonist and antioxidant activities. Also, the effect was further investigated *in vivo* using rats fed a high-fat and high-fructose diet supplemented with safflomide.

■ MATERIALS AND METHODS

Materials. Tryptamine, caffeic acid, coumaric acid, cinnamic acid, dichloromethane, 1,3-diisopropylcarbodiimide, ketanserin, 5-HT agonists, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (BRL Life Technologies, Grand Island, NY).

Methods. *Syntheses of Safflomide and Analogues.* Safflomide, *N*-coumaroyltryptamine, and *N*-cinnamoyltryptamine

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were synthesized as described previously.[26,27] Briefly, respective acids (caffeic, coumaric, and cinnamic acids) were dissolved in dichloromethane (DCM) and converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide (DIC). Then, tryptamine was added to the reaction mixture and stirred gently for 3 h. The synthesized products were recovered and purified by high-performance liquid chromatography (HPLC, Waters, Milford, MA).[27]

Cell Culture. 3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as described previously.[28] Briefly, cells were cultured in DMEM (GIBCO, Grand Island, NY) containing 10% bovine calf serum (BCS) until confluent. At 2 days after confluence, the cells were stimulated to differentiate in DMEM containing 10% fetal bovine serum (FBS), by treatment with 167 nM insulin, 0.5 μM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone for 2 days. Cells were then maintained in the same DMEM containing 167 nM insulin for another 2 days, followed by culturing with the DMEM for an additional 4 days, at which time more than 90% of cells were mature adipocytes with accumulated fat droplets. All media contained 100 units/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine (Invitrogen, Carlsbad, CA). Safflomide, safflomide analogues, ketanserin, and 5-HT agonists were dissolved in ethanol and added to cultures of 3T3-L1 cells prepared as described above after 4 days (the final concentration of ethanol was less than 0.1%). 3T3-L1 cells were treated with 5-HT2A agonists (serotonin and α-Me-5-HT) for 30 min prior to the treatment with safflomide. The cells were cultured for an additional 4 more days, harvested, and used for mRNA extraction and the adiponectin enzyme-linked immunosorbent assay (ELISA).

RNA Isolation and Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) synthesized from 2 μg of total RNA using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Inc., Santa Clara, CA) according to the protocol of the manufacturer. Real-time quantitative PCR was carried out using TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems, Foster City, CA) and ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) following the protocol of the manufacturer. Mouse TaqMan probes and primers were purchased from Applied Biosystems using inventoried TaqMan gene expression assays: adiponectin (assay ID Mm00456425_m1), leptin (assay ID Mm00434759_m1), interleukin-6 (assay ID Mm00446190_m1), CCL2/MCP-1(assay ID Mm00441242_m1), and TNF-α (assay ID Mm00443258_m1). Mouse TATAA binding protein (assay ID Mm00446973_m1) was used as an endogenous control. Quantitation of mRNA levels was performed using the ΔCt method.[30]

Measuring Adiponectin Production in 3T3-L1 Cells. Cultured 3T3-L1 cells were washed with PBS and suspended in a lysis buffer [50 mM Tris at pH 7.5, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF)]. The cell extract were prepared via mixing 3T3-L1 cells with the lysis buffer followed by a brief sonication. Adiponectin in the extract was measured using a mouse adiponectin ELISA kit (Millipore Corp., Billerica, MA).

Animal Study. Sprague−Dawley, 8-week-old, male rats (weighing 125−155 g) were purchased from Charles River (Wilmington, MA). Rats were acclimated to the experimental facility for at least 2 weeks and housed in ventilated microisolator racks with an automatic watering system in a room with a 12/12 h light/dark cycle, ambient temperature of 23−18 °C, and relative humidity of 55.5%. All animal procedures were approved by the Beltsville Area Animal Care and Use Committee. Rats were fed an AIN-76A purified diet, providing the recommended allowance of all nutrients required for maintaining optimal health. Rats in the experimental trial were divided into three groups and fed a control (NC) diet, a high-fructose and high-fat diet (HFD), or a high-fructose and high-fat diet with safflomide (3 mg/day) (HFDS) (n = 10) for 12 weeks. The composition of the NC diet was based on the AIN-76 diet. The HFD and HFDS diets were prepared by substituting 60% of the carbohydrate in the NC diet with 20% fat and 40% fructose. In addition, the HFDS group received safflomide via drinking water to rats on the HFD diet (HFDS group). The energy levels of the HFD and HFDS diets were 4228 kcal/kg, while that of the NC group was 3679 kcal/kg. The rats were given food and distilled water *ad libitum* during the experimental period. Food consumption and weight gain were measured daily and weekly, respectively. At the end of the experimental period, rats were sacrificed following a 12 h fast, blood samples were collected in EDTA-coated tubes and centrifuged (3000 rpm for 15 min), and the separated plasma used to determine glucose, insulin, and adipokine levels. The visceral fat was also surgically removed and weighed.

Plasma Adiponectin, Insulin, and Glucose Measurement. Plasma adiponectin was detected using a rat adiponectin ELISA kit (Millipore Corp., Billerica, MA). Insulin was detected using a rat/mouse insulin ELISA kit (Millipore Corp., Billerica, MA). Glucose was detected using a blood glucose meter (Bayer, Tarrytown, NY).

Insulin Resistance Assay. The degree of insulin resistance was estimated by a homeostasis assessment model (HOMA-IR) calculated by the following formula:

$$\text{HOMA-IR} = \text{plasma glucose (mg/dL)} \times \text{serum insulin (mU/L)} / 405$$

Insulin values were expressed in international units (1 IU = 0.041 67 mg).[13,14]

Statistical Analysis. GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) software was used for statistical analysis. Multiple group data were analyzed using analysis of variation (ANOVA) followed by posthoc analysis with the Bonferroni test. The unpaired Student's t test was used to compare means between the two groups. Values were considered significant at $p < 0.05$. Statistically significant differences are defined at the 95% confidence interval. Data are shown as the mean ± standard deviation (SD).

■ RESULTS AND DISCUSSION

Effect of Safflomide on the Expressions of Adipokines in 3T3-L1 Cells. During the differentiation of 3T3-L1 preadipocytes to adipocytes, the cells produce a wide range of adipokines affecting metabolism and the function of many organs and tissues, including muscle, liver, vasculature, and brain.[11,12] In this study, we investigated the effects of safflomide on the expression of adiponectin, IL-6, and MCP-1/CCL2. We found that treatment of 3T3-L1 cells with 20 μM safflomide led to more than 2-fold change in adiponectin mRNA expression (Figure 1). This is a surprising result because adiponectin levels typically decrease with an increased hypertrophy of adipocytes, commonly observed in obesity.[11−14] However, we did not observe changes in IL-6 and MCP-1 (Figure 1).

Dose-Dependent Effect of Adiponectin Expression in 3T3-L1 Cells Induced by Safflomide. We investigated dose-dependent effects of safflomide on adiponectin mRNA expression in 3T3-L1 cells. The cells were treated with four different concentrations of safflomide (1, 5, 10, and 20 μM), and adiponectin expression was determined. Safflomide increased adiponectin mRNA expression in a dose-dependent manner, although the upregulation was reduced at 20 μM (Figure 2). Between 1 and 20 μM, adiponectin expression was upregulated by more than 5-fold (p < 0.015), with the greatest upregulation observed at 10 μM (Figure 2). Consistent with upregulation of adiponectin mRNA, adiponectin protein was upregulated in safflomide-treated 3T3-L1 adipocytes (Figure 3).

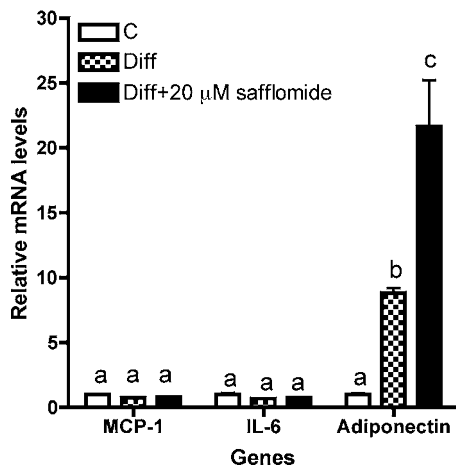

Figure 1. Effect of safflomide on adipokines in 3T3-L1 cells. Cells were treated with 20 μM safflomide; RNA was isolated; and gene expression of MCP-1, IL-6, and adiponectin was determined using real-time PCR. Results are expressed as the mean ± SD (n = 3): (white bar) control 3T3-L1 cells, (hatched bar) 3T3-L1 cells induced to fat cells, and (black bar) 3T3-L1 cells treated with 20 μM safflomide and induced to fat cells. Data were analyzed using ANOVA, as described in the Materials and Methods. (*) Significant difference (p < 0.05) from the vehicle control culture.

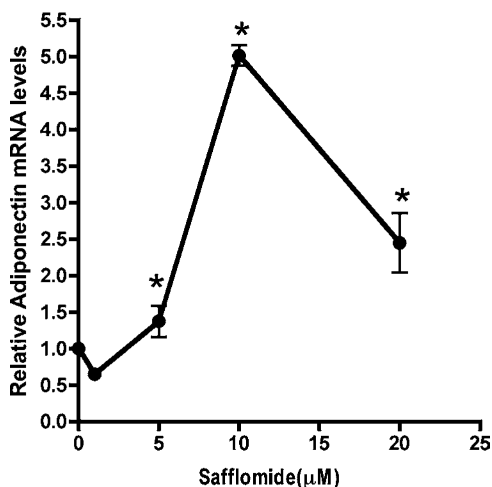

Figure 2. Concentration-dependent effects of safflomide on adiponectin expression. 3T3-L1 cells were treated with 0, 1, 5, 10, and 20 μM safflomide; RNA was isolated; and gene expression of adiponectin was determined using real-time PCR. Results are expressed as the mean ± SD (n = 3). Data were analyzed using ANOVA, as described in the Materials and Methods. (*) Significant difference (p < 0.05) from the vehicle control culture.

Effects of Serotonin Receptor Antagonist Activity of Safflomide on Adiponectin Production in 3T3-L1 Cells. Recent studies suggested that the treatment of 5-HT2A receptor antagonists increased adiponectin expression in hypertrophic 3T3-L1 cells.[28,29] Therefore, we investigated the effect of serotonin receptor antagonist activity of safflomide on adiponectin production in 3T3-L1 cells. The protein level of adiponectin, rather than its mRNA, was measured because increased adiponectin protein is a biological manifestation of upregulated mRNA expression. We hypothesized that safflomide induced adiponectin production in 3T3-L1 cells by blocking 5-HT2A receptors, similar to the 5-HT2A antagonist ketanserin, and that 5-HT2A agonists, such as serotonin and α-

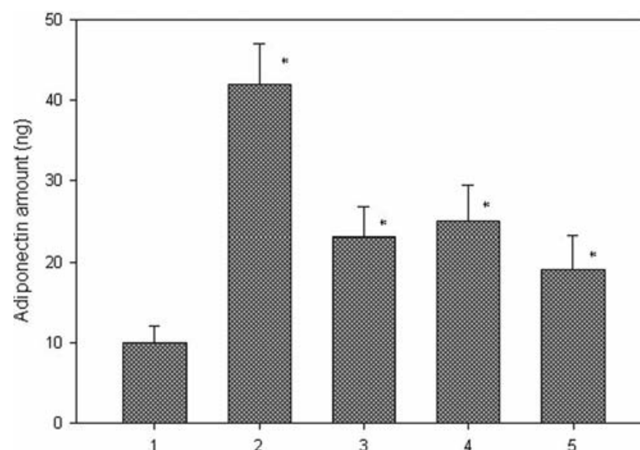

Figure 3. Serotonin receptor antagonist activity of safflomide on adiponectin production in 3T3-L1 cells: (1) positive control, (2) safflomide, (3) safflomide + serotonin, (4) safflomide + α-Me-5-HT, and (5) ketanserin. Data are shown as the mean ± SD (n = 9) of four samples. Data were analyzed using one-way ANOVA, as described in the Materials and Methods. (*) Significant difference (p < 0.05) from the vehicle control culture.

Me-5-HT, should be able to suppress safflomide-induced adiponectin production. As shown in Figure 3, treatment of 3T3-L1 cells with serotonin and α-Me-5-HT reduced adiponectin production, while ketanserin increased adiponectin production, although the level of increase is less than with safflomide. These data suggest that safflomide may increase adiponectin production by more than a blocking of 5-HT2A receptors on 3T3-L1 cells.

Safflomide has serotonin receptor antagonist activity as well as antioxidant activity, which ketanserin lacks.[26,27] Therefore, we investigated the auxiliary role of antioxidant activity in adiponectin production using two safflomide analogues (N-cinnamoyltryptamine and N-coumaroyltryptamine). Similar to safflomide, N-cinnamoyltryptamine and N-coumaroyltryptamine have non-selective 5-HT receptor antagonist activity but less antioxidant activity because they lack 3,4- and 4-hydroxylation in the benzene ring. As expected, they increased adiponectin production in 3T3-L1 cells but to a less extent than safflomide (Figure 4). To further demonstrate that this decrease is due to the lack of 3,4-hydroxylation of the caffeic acid moiety of safflomide, we treated 3T3-L1 cells with caffeic acid in the same way that they were treated with safflomide. As shown in Figure 4, caffeic acid was able to increase adiponectin production much less than safflomide, suggesting that the antioxidant activity of safflomide may play only a modest role in increasing adiponectin production. These data indicate that safflomide may increase adiponectin production in 3T3-L1 cells both by blocking 5-HT2A receptors as well as through antioxidant activity.

Effect of Safflomide on Adiponectin Expression *in Vivo*. Having observed that safflomide was capable of increasing adiponectin production in 3T3-L1 cells *in vitro*, we developed a rat model to test its activity *in vivo*. Rats were fed either a control NC diet, a high-fat and high-fructose (HFD), or a high-fat and high-fructose diet supplemented with safflomide (HFDS). Safflomide (3 mg/day) was provided to rats via drinking water because a preliminary study indicated that safflomide exhibited good bioavailability with a C_{max} of 1−2 μM after oral administration.[27] Because adiponectin has antidiabetic, antiatherogenic, and anti-inflammatory activities[4,5]

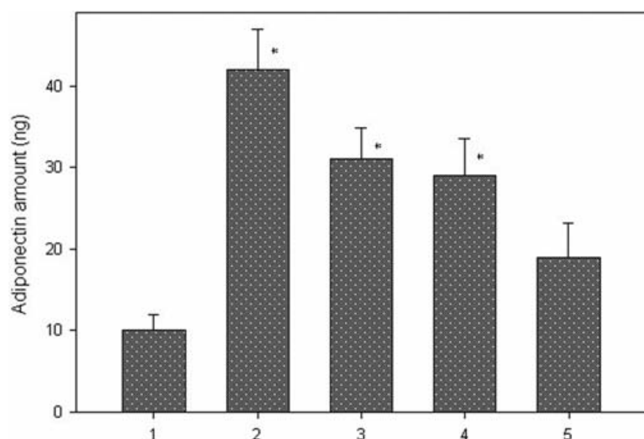

Figure 4. Antioxidant activity of safflomide on adiponectin production in 3T3-L1 cells: (1) positive control, (2) safflomide, (3) *N*-cinnamoyltryptamine, (4) *N*-coumaroyltryptamine, and (5) caffeic acid. Data are shown as the mean ± SD ($n = 9$) of four samples. Data were analyzed using one-way ANOVA, as described in the Materials and Methods. (*) Significant difference ($p < 0.05$) from the vehicle control culture.

and is negatively correlated with obesity and insulin resistance,[5,6] we determined adiponectin levels in rats following feeding the NC, HFD, and HFDS diets for 12 weeks. The rats fed the HFDS diet showed 30% ($p < 0.039$) and 60% ($p < 0.027$) higher plasma adiponectin levels than rats fed the NC and HFD diets, respectively (Table 1). We also measured plasma concentrations of safflomide and found the range of the plasma concentration between 1 and 3 μM that was expected on the basis of our previous study.[27] These data clearly indicate that safflomide (3 mg) supplementation increased production of plasma adiponectin in rats.

Table 1. Effects of the Safflomide Supplementation on Bodyweight, Visceral Fat, and Plasma Adiponectin Levels in Rats[a]

	NC	HFD	HFDS
total body weight (g)	600 ± 45	657 ± 39 a	628 ± 37
visceral fat (g)	20.7 ± 1.4	29.6 ± 1.2 a	24.2 ± 1.3 b
adiponectine (ng/mL)	39.1 ± 4.3	32.5 ± 3.7 a	51.9 ± 4.7 b

[a]NC is a control diet group; HFD is a high-fat and high-fructose diet group; and HFDS is a high-fat and high-fructose diet group supplemented with safflomide. Data were analyzed using ANOVA, as described in the Materials and Methods. Data are shown as the mean ± SD ($n = 10$). The letter a indicates statistical significance compared to the NC diet group ($p < 0.05$), and the letter b indicates statistical significance compared to the HFD diet group ($p < 0.05$).

Effects of Safflomide on Body Weight and Visceral Fat. The body weight gain of rats fed the HFD diet was significantly higher than rats fed the NC or HFDS diets (Table 1), although the initial body weights and feed intakes of rats on the three diet groups were not significantly different (data not shown here). These data indicate that safflomide exerted a positive effect on weight reduction in rats fed a high-fat and high-fructose diet. We investigated whether the change in body weight gain was related to visceral fat. The average weight of the visceral fat of rats fed the HFD diet was more than 40% higher than rats fed the NC diet, while the average fat weight of rats fed the HFDS diet was less than 20% higher than that of rats fed the NC diet ($p < 0.05$). These data suggest that safflomide supplementation significantly lowered visceral fat weight and body weight in rats fed a HFD diet.

Improved Insulin Resistance Induced by Safflomide. Because increased plasma adiponectin is strongly associated with insulin sensitivity and/or insulin resistance,[15,16] we investigated the effect of safflomide supplementation on insulin resistance related to adiponectin expression in rats fed the three diets for 12 weeks. The level of insulin resistance was estimated by a homeostasis assessment model (HOMA-IR), described in the Materials and Methods. As shown in Table 2, average plasma glucose concentrations were comparable (approximately 126 mg/dL) in all three groups following the 12 weeks of feeding but average plasma insulin levels for each group were significantly different: NC (36 950 mU/L), HFD (45 830 mU/L), and HFDS (39 830 mU/L). The average plasma insulin level of rats fed the HFDS diet was significantly lower than that of rats on the HFD diet, although the level was still a bit higher than rats on the NC diet. When converted to HOMA-IR units, a commonly used means to assess insulin sensitivity,[31] the HOMA-IR value of rats on the HFDS diet was significantly lower than that of rats on the HFD diet, suggesting an improved insulin sensitivity ($p < 0.05$) (Table 2).

Table 2. Effects of the Safflomide Supplementation on Blood Glucose, Insulin, and HOMA-IR in Rats[a]

	glucose (mg/mL)	insulin (mU/L)	HOMA-IR
NC	127 ± 5.7	36950 ± 3247	11586 ± 1252
HFD	126 ± 4.5	45830 ± 4223 a	14258 ± 1613 a
HFDS	126 ± 5.1	39830 ± 4176 b	12391 ± 1289 b

[a]NC is a control diet group; HFD is a high-fat and high-fructose diet group; and HFDS is a high-fat and high-fructose diet group supplemented with safflomide. Data were analyzed using ANOVA, as described in the Materials and Methods. Data are shown as the mean ± SD ($n = 10$). Data were considered as significant when $p < 0.05$. The letter a indicates statistical significance compared to the NC diet group ($p < 0.05$), and the letter b indicates statistical significance compared to the HFD diet group ($p < 0.05$).

All together, the data suggested that safflomide is a potent plant-derived amide able to increase adiponectin production related to reduced visceral fat accumulation and improved insulin resistance in rats fed the HFD diet.

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Notes

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The authors declare no competing financial interest.

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