Raspberry Ketone Increases Both Lipolysis and Fatty Acid Oxidation in 3T3-L1 Adipocytes

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Key words
- adiponectin
- β-oxidation
- lipid metabolism
- herbal medicine
- raspberry ketone

Abstract

Raspberry ketone (RK) is a natural phenolic compound of the red raspberry. The dietary administration of RK to male mice has been reported to prevent high-fat diet-induced elevation in body weight and to increase lipolysis in white adipocytes. To elucidate a possible mechanism for the antiobesity action of RK, its effects on the expression and the secretion of adiponectin, lipolysis, and fatty acid oxidation in 3T3-L1 were investigated. Treatment with 10 µM of RK increased lipolysis significantly in differentiated 3T3-L1 cells. An immunoassay showed that RK increased both the expression and the secretion of adiponectin, an adipocytokine mainly expressed and secreted by adipose tissue. In addition, treatment with 10 µM of RK increased the fatty acid oxidation and suppressed lipid accumulation in 3T3-L1 adipocytes. These findings suggest that RK holds great promise as an herbal medicine since its biological activities alter the lipid metabolism in 3T3-L1 adipocytes.

Introduction

Obesity is a complex metabolic disorder resulting from an imbalance between energy intake and expenditure. This disregulation may have a genetic or behavioral origin, involving the quality and quantity of food intake as well as lifestyle. Obesity is characterized by an increase in body weight and adipose tissue hyperplasia and hypertrophy with excessive fat storage [1].

Adiponectin is an adipocytokine mainly expressed and secreted by adipose tissue that has been shown to regulate lipid and glucose metabolism and to play a key role in body weight regulation and homeostasis. Adiponectin has also been reported to be involved in obesity and with associated metabolic diseases, its concentration being decreased in these conditions [2,3]. Administration of adiponectin to obese or diabetic mice reduces circulating free fatty acid levels by enhanced skeletal muscle fat oxidation [4–6]. Lipolysis and fatty acid oxidation in adipose tissue might also be important mechanisms involved in reducing body fat.

Raspberry ketone [4-(4-hydroxyphenyl) butan-2-one; RK], a major aromatic compound contained in the red raspberry (*Rubus idaeus* L.), is widely used as a fragrance in cosmetics and as a flavoring agent in foodstuffs [7]. RK has a structure similar to those of capsaicin and synephrine (**Fig. 1**) [8]. Capsaicin and synephrine have been reported to exert a lipolytic activity in rats fed a high-fat diet and fat cells, respectively [9,10]. In the view of structural similarities, it is possible that RK has a lipolytic activity in adipocytes. Furthermore, when given to male mice in high doses, RK has been shown to prevent the high-fat diet-induced elevations in body weight and increase lipolysis in white adipocytes [11]. Despite reports on the effects of RK on lipid metabolism, the underlying mechanism of RK action has not been thoroughly understood.

In this study, to understand a possible mechanism for the antiobesity action of RK, its effects on the expression and the secretion of adiponectin, lipolysis, and fatty acid oxidation in 3T3-L1 were examined.

Material and Methods

Reagents

RK with 99.9% purity and naringenin (98.0% purity) were purchased from Aldrich Chemical Co. Isoproterenol (98.0% purity), insulin, dexamethasone, 3-isobutyl-1-methylxanthine, and forskolin

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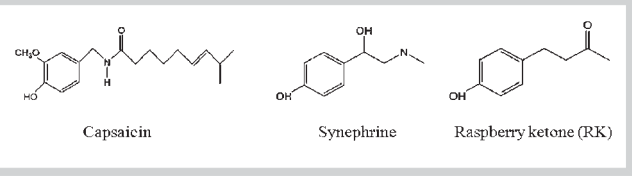

Fig. 1 Structural formulas of capsaicin, synephrine, and raspberry ketone.

were from Sigma. Auraptene (≥ 98.0% purity) was purchased from LKT Lab. All tissue culture materials were from GIBCO. The enhanced chemiluminescence (ECL) detection kit and horseradish peroxide-conjugated secondary antibodies were from Amersham Pharmacia Biotech.

3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes (American Type Culture Collection ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (10 000 U/mL penicillin and 10 000 cg/mL streptomycin in 0.85% saline), and 1% (v/v) 100 µM pyruvate at 37 °C in 95% air 10% CO_2. Differentiation of 2-day postconfluent preadipocytes (designated as day 0) was initiated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 1 µg/mL insulin in DMEM supplemented with 10% fetal bovine serum. After 48 hr (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 µg/mL insulin, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. RK was reconstituted, filter sterilized, and stored at − 20 °C. For each experiment, cells received RK premixed with culture medium. Unless otherwise noted, "vehicle" refers to dimethyl sulfoxide (DMSO) in culture medium or methylisobutylxanthine, dexamethasone, and insulin (MDI) differentiation medium.

Measurement of lipolysis

Lipolytic activity was measured by assaying glycerol released from cells into incubation buffer. Briefly, on days 8 to 12, the medium was removed and the differentiated adipocytes were incubated for 24 hr in low-glucose DMEM containing 2% (w/v) fatty acid-free bovine serum albumin with various concentrations of RK. The incubation mixture was aspirated and used to assay glycerol. Glycerol content was determined using a colorimetric assay (GPO-Trinder; Sigma-Aldrich), and protein content was measured using a BCA protein assay.

Western blot analysis of adiponectin expression

Differentiated 3T3-L1 cellular proteins were dissolved in the lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 10 mM EDTA, 100 mM NaCl, and 1% β-mercaptoethanol) containing protease inhibitors, and centrifuged at 12 000 g for 15 min at 4 °C. The protein concentrations were determined by BCA protein assay kit (Pierce). 3T3-L1 adipocyte cellular protein extracts (10 µg) were analyzed on the Novex precast 4 to 20% Tris-glycine-SDS-polyacrylamide gels and transferred on polyvinylidene difluoride membranes as indicated by the manufacturer. The following steps were performed at room temperature. The membranes were blocked with TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 2 hr and were then incubated for 3 hr with specific antibodies rabbit anti-mouse adiponectin (R&D System) in TBS-T containing 2% nonfat dry milk. After three 15-min washes in TBS-T, membranes were incubated for 40 min with horseradish peroxidase-conjugated anti-rabbit antiserum and then washed three times for 15 min in TBS-T. The immunoreactivity was revealed with the ECL-plus chemiluminescent substrate.

Determination of adiponectin secretion by ELISA

Adiponectin in cell culture supernatants was estimated by quantification with the use of ELISA kits (R&D Systems). The media of cells exposed or not to various concentrations of RK were centrifuged for 5 min at 500 g and the supernatants were subsequently diluted for use in the quantification reaction. Adiponectin levels were determined according to the manufacturer's instructions.

Measurement of fatty acid oxidation

The rate of cellular β-oxidation of [9,10(n)-^{3}H]palmitic acid (52 Ci/mmol; Amersham) was measured as ^3H_2O release. The reaction in differentiated 3T3-L1 cells was carried out in triplicate (800 µL per well in 6-well culture plates), each containing 2 µCi [9,10(n)-^{3}H]palmitic acid, which was suspended in Hanks' balanced salt solution (HBSS) containing 5 mg/mL fatty acid-free BSA. After 4 hr incubation at 37 °C under humidified air containing 10% CO_2 with various concentrations of RK, 600 µL of the reaction medium was transferred to new Eppendorf tubes containing 600 µL of 10% (w/v) trichloroacetic acid. After 2 min at room temperature, the reaction mixture was centrifuged at 8500 g for 5 min. 600 µL of the supernatants were immediately removed, then 870 µL of methanol/chloroform (2 : 1) and 360 µL of 2 M KCl/2 M HCl were added. After vigorously mixing the reactions, mixtures were centrifuged at 3000 *g* for 5 min, and the aqueous phase, 600 µL, containing ^3H_2O, was transferred to a new tube, treated once more with the methanol/chloroform and KCl/HCl mixture, recovered, and radioactivity was measured.

Oil-red O staining

On day 8 of adipocyte differentiation induction, the cells were stained with Oil-red O dye (Sigma-Aldrich Chemical Co.). The cells were fixed with 70% ethanol, dehydrated with 100% propylene glycol and stained with Oil-red. Fat droplets in adipocytes were stained red. To quantify retention of Oil-red O, stained adipocytes were extracted with 1 mL of 4% Igepal CA-630 (Sigma) in isopropanol for 15 min, and absorbance was measured by spectrophotometry at 520 nm.

Cytotoxicity assay

For the cytotoxicity study, 3T3-L1 preadipocytes were cultured and differentiated as described above. After RK treatment for 4 days in DMEM containing 10% fetal bovine serum, lactate dehydrogenase (LDH) concentration in the medium was immediately detected with the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega).

Statistics

Data expressed as mean ± SD were obtained from three separate experiments. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnett's test, as appropriate; $p < 0.05$ was considered statistically significant.

Table 1 Effect of RK on 3T3-L1 adipocyte lipolysis.

Treatment	Glycerol released to medium (μg/mL)
Control (vehicle)	15.63 ± 3.49
Isoproterenol (1 μM)	28.66 ± 5.47*
RK (1 μM)	19.96 ± 8.08
RK (10 μM)	49.84 ± 5.44*

Values are mean ± SD of three separate experiments and are expressed as the amount of glycerol released to medium. Two-day postconfluent 3T3-L1 cells were differentiated according to the MDI protocol. Fully differentiated cells were treated for 24 hr with 1 or 10 μM RK. The conditioned medium was then removed from each well and assayed for glycerol content. Isoproterenol was used as a positive control. * $P < 0.05$, significantly different from vehicle

Results

To determine whether RK exerts lipolytic effects in 3T3-L1 cells, 10 μM of RK was treated with 3T3-L1 adipocytes (days 8–10 after MDI stimulation). As shown in **Table 1**, 10 μM of RK induced a 3-fold greater release of glycerol into the culture medium than did the control (vehicle).

It was also investigated whether RK affects the expression and the secretion of adiponectin in differentiated 3T3-L1 adipocytes. Four days of treatment of subconfluent cultures of 3T3-L1 preadipocytes with 10 μM of RK increased markedly both the cellular (**Fig. 2a**) and the secreted levels (**Fig. 2b**) of adiponectin. For the immunoblot analysis of adiponectin expression levels, auraptene was used as a positive control [12]. Treatment with 10 μM of RK led to an increase of 77 percent in the band intensity of adiponectin compared to the negative control (**Fig. 2a**). As shown in **Fig. 2b**, the secretion levels of adiponectin were significantly upregulated by the RK treatment (10 μM).

To evaluate the effect of RK on fatty acid oxidation, the oxidation levels of labeled palmitic acids were assessed in 3T3-L1 cells. The degree of oxidation of palmitic acid was significantly higher in cells pretreated with 10 μM RK than in control cells (**Fig. 3**).

To examine the effect of RK on the triglyceride content of 3T3-L1 cells, cultured 3T3-L1 adipocytes were exposed to 10 μM RK (at day 0), and cell differentiation was performed with a differentiation medium. At day 8, differentiation was terminated and fat drops were detected by Oil-red O staining (**Fig. 4a**). As shown in **Fig. 4b**, treatment of 3T3-L1 cells with 10 μM RK markedly suppressed lipid accumulation.

To determine whether RK increased both lipolysis and fatty acid oxidation through cytotoxic effects, LDH release into the culture medium in response to RK treatment was measured. Effect of Triton X-100 (0.1%) on LDH release was examined as a positive control [13]. Up to the concentration of 100 μM, RK did not increase LDH release (**Fig. 5**). This result suggests that RK did not exhibit toxicity in this experimental system.

Discussion

In plants, RK is synthesized from coumaroyl-CoA. But since the natural abundance of RK is very low, it is prepared industrially by a variety of methods from chemical intermediates [14].

Based on its structural similarities to capsaicin and synephrine (**Fig. 1**), it could be hypothesized that RK might affect the lipid metabolism in similar ways to these compounds. Capsaicin, a pungent principle of hot red pepper, has been reported to de-

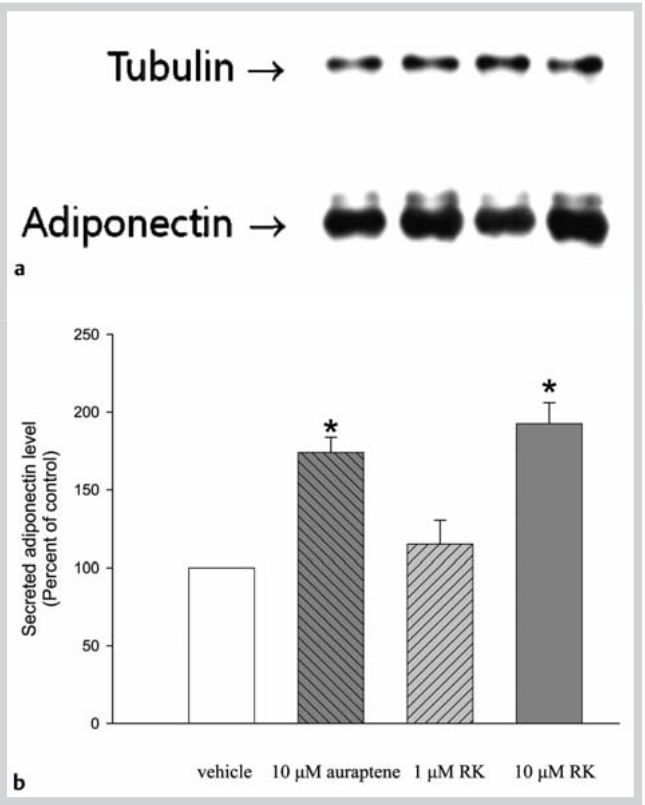

Fig. 2 Effect of RK on adiponectin protein levels in cellular extract and in conditioned medium of cultured 3T3-L1 adipocytes. Subconfluent 3T3-L1 preadipocytes were exposed or not (control) to increasing concentrations of RK (1 or 10 μM) added every day. After 4 days of treatment, the medium was changed and cell cultures were exposed for an additional 24 hr to serum-free medium. Cellular protein extracts (200 μg) (**a**) and secreted proteins from conditioned medium (24 hr) (**b**) were assayed by Western blotting and ELISA, respectively. Values represent the mean ± SD of the percentage of adiponectin secretion in cultures treated with vehicle only from three independent experiments. 27.5 ng/mL of adiponectin was taken for 100% (**b**). * $P < 0.05$ compared with control (vehicle).

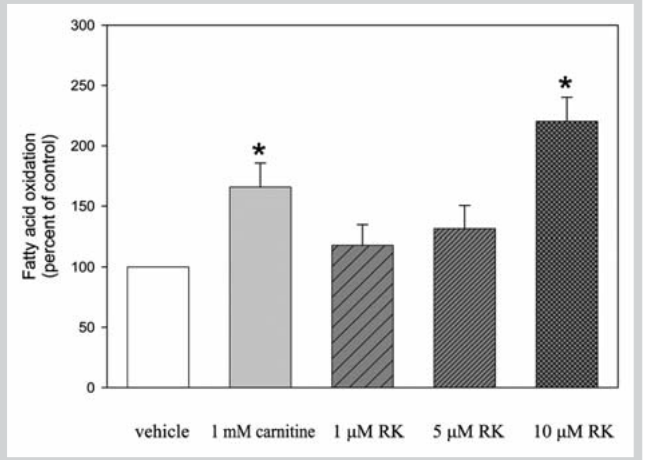

Fig. 3 Effect of RK on fatty acid oxidation in 3T3-L1 cells. The harvested cells were incubated at 37 °C for 4 hr in the presence of various concentrations (1 to 10 μM) of RK in triplicates. The rate of cellular β-oxidation of [9,10(n)-^{3}H]palmitic acid (52 Ci/mmol; Amersham) was measured as ^3H_2O release. All values were expressed as mean ± SD of three separate experiments. * $P < 0.05$ compared with control (vehicle).

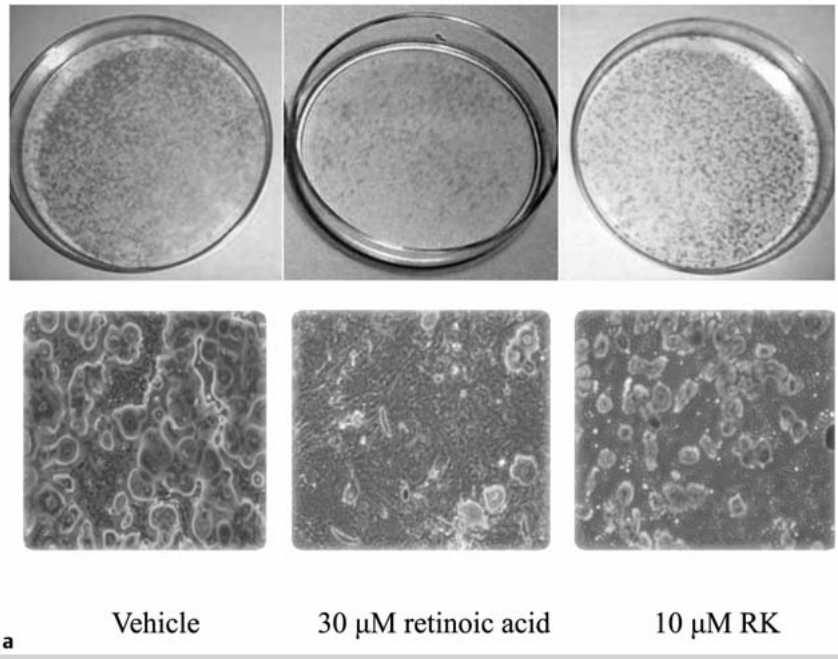

Fig. 4 a Effect of RK on the triglyceride content of 3T3-L1 cells. 3T3-L1 cells pretreated with 10 µM of RK were grown at 37 °C for 4 hr (at day 0). Cells were stained with Oil-red O to determine the triglyceride content of the cells (at day 8). The images shown are representative of two independent experiments (upper panel). Microscopic view (200×) of selected dishes shown in upper panel (bottom panel).

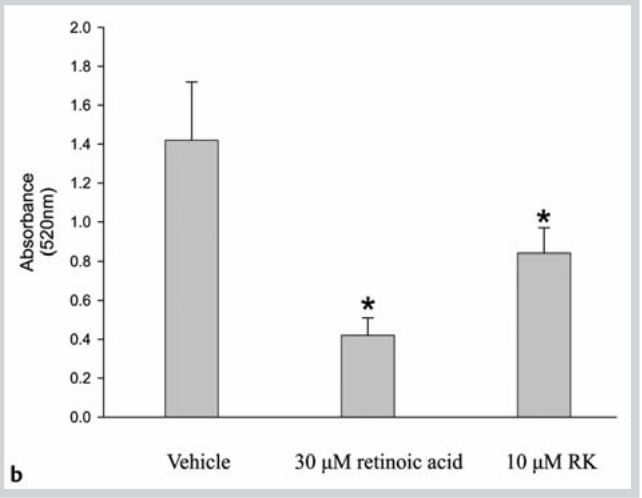

Fig. 4 b Effect of RK on the triglyceride content of 3T3-L1 cells. To estimate the accumulation of neutral lipid, Oil-red O was extracted with 4% Igepal in isopropanol and quantified with spectrophotometry at 520 nm. The results are from two independent experiments and are presented as the means ± SD. * $P < 0.05$ compared with control (vehicle).

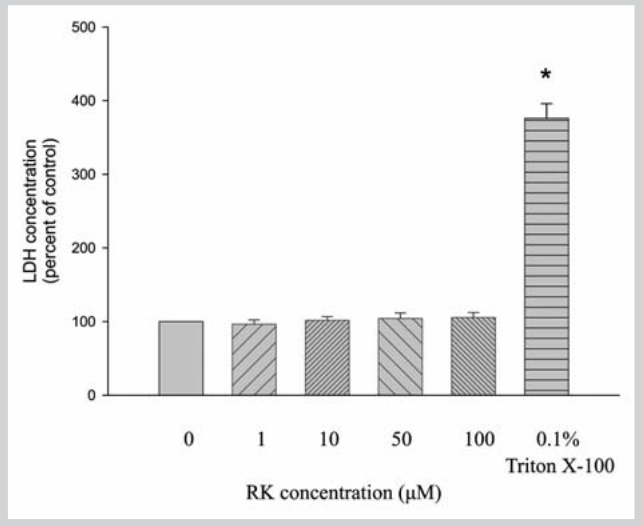

Fig. 5 RK had no cytotoxicity in 3T3-L1 cells. 3T3-L1 preadipocytes were exposed or not (control) to increasing concentrations of RK (1 to 100 µM). After RK treatment for 4 days in DMEM containing 10% fetal bovine serum, lactate dehydrogenase (LDH) concentration in the medium was immediately detected. The treatment of Triton X-100 (0.1%) for 30 min was used as a positive control. Values represent the mean ± SD of the percentage of LDH release of cultures treated with vehicle only from three independent experiments. * $P < 0.05$ compared with control (vehicle).

crease the adipose tissue weight and serum triacylglycerol content by enhancing energy metabolism [9]. Synephrine, a compound found in citrus plants, exerts a lipolytic activity in fat cells [10]. The present study demonstrated that RK affected the lipid metabolism by stimulating lipolysis, fatty acid oxidation, and the secretion of adiponectin in 3T3-L1 adipocytes (**Table 1**, **Figs. 2** and **3**), which is consistent with the report by Morimoto et al. that RK has an antiobesity function in rodents [11]. Given the potent induction of lipolysis and fatty acid oxidation in 3T3-L1 cells, RK might help to reduce the fat mass in adipocytes. The validity of this hypothesis was demonstrated by Oil-red O staining, since treatment of 3T3-L1 cells with 10 µM RK markedly suppressed lipid accumulation (**Fig. 4**).

Adiponectin is one of the most important adipocytokines and a regulatory factor in obesity and insulin resistance. The increase of adiponectin secretion is regarded as a target for drug discovery and therapy of obesity-related metabolic diseases. This study showed that a four-day treatment of subconfluent cultures of 3T3-L1 preadipocytes with 10 µM of RK increased both the cellular and the secreted levels of adiponectin, a late marker of adipocyte cell maturation (**Fig. 2 a** and **b**).

The AMP-activated protein kinase (AMPK) pathway was identified as the signaling cascade through which leptin activates lipid oxidation in skeletal muscle [15]. The activation of AMPK results in the phosphorylation of several target molecules and consequent stimulation of fatty acid oxidation, glucose transportation in muscle, and cardiac glycolysis [16, 17]. Considering the stimulatory effect of RK on fatty acid oxidation, a further study will be needed to investigate whether the AMPK pathway is involved in the RK-stimulated fatty acid oxidation.

In conclusion, the effects of RK on lipolysis, fatty acid oxidation, and adiponectin secretion were confirmed in 3T3-L1 adipocytes. These findings might help to explain the underlying mechanisms in the reported alteration of the lipid metabolism in RK-treated mice [11].

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