Rutin Stimulates Adipocyte Differentiation and Adiponectin Secretion in 3T3-L1 Adipocytes

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Rutin is a flavonoid, which is found in many plants. It has been shown to reduce blood glucose and increase insulin levels in diabetic rats. In the present study, the authors aimed to elucidate the molecular basis for the observed antidiabetic activity using murine 3T3-L1 preadipocyte cultures. The treatment of differentiating 3T3-L1 cells with rutin at concentrations of 3, 10, 30 and 100 μM significantly increased lipid accumulation and mRNA expression of transcription factors, such as peroxisome proliferator-activated receptor gamma, CCAAT/enhancer-binding protein alpha, and adipocyte fatty acid-binding protein. Furthermore, rutin at concentrations of 10, 30 and 100 μM increased adiponectin mRNA expression together with stimulating the secretion of adiponectin in differentiating 3T3-L1 cells. These results indicate that the stimulatory effect of rutin on adipocyte differentiation likely occurs through up-regulation of adipogenic transcription factors and downstream adipocyte-specific gene expression. Such effects of rutin on adiponectin secretion and adipocyte activity may account for, at least in part, the antidiabetic effects of consumption of food containing rutin.

Keywords: *Rutin, Adipogenesis, Adiponectin, 3T3-L1 adipocytes*

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Adipose tissue is an important metabolic organ that is crucial for whole-body insulin sensitivity and energy homeostasis. During adipogenesis, fibroblast-like preadipocytes differentiate into lipid-laden and insulin-responsive adipocytes. Many of the transcription factors including peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) have been shown to play significant role in promoting adipogenesis[1]. There is an evidence shows that activation of PPARγ promotes adipocyte differentiation as well as increases insulin sensitivity[2]. As the cells differentiate to adipocyte, they become spherical, accumulate lipid droplets, and express the characteristic of adipogenic markers such as adipocyte fatty acid binding protein (aP2)[3], and adiponectin[4].

Rutin is a flavonoid of the flavonol type. It was reported that rutin has several pharmacological properties including antioxidant, anticarcinogenic, cytoprotective, antiplatelet, antithrombic, vasoprotective and cardioprotective activities[5-9]. As described above, adipogenesis, particularly because of the involvement of PPARγ stimulation and adiponectin secretion, is an important physiologic process that may ameliorate insulin resistance in type 2 diabetes mellitus. Therefore, the authors have the objective to determine the effect of rutin on the adipogenic process in 3T3-L1 preadipocytes by investigating the actions of rutin on (a) lipid accumulation; (b) expression of the adipogenic transcription factors, C/EBPα and PPARγ, and of a key marker for adipogenesis, aP2 and adiponectin, and (c) adiponectin secretion.

Material and Method

Chemical reagents

Mouse 3T3-L1 preadipocytes were kindly provided by Dr Kim Jae-Woo (Yonsei University College of Medicine, South Korea). Dulbecco modified Eagle medium/high glucose (DMEM/HG) was purchased from HyClone Laboratories (Logan, Utah, USA). QIAzol Lysis Reagent was purchased from Qiagen Science (Madison, Mich, USA). All other chemicals used were from Sigma-Aldrich (St. Louis,

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3T3-L1 adipocyte culture

The 3T3-L1 preadipocytes were plated in 24-well plates at a density of $2x10^5$ cells/well and cultured in DMEM containing 25 mmol/L glucose (DMEM/HG), 1 mmol/L sodium pyruvate, 4 mmol/L l-glutamine, 10% calf bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO_2. To induce differentiation at 2 days after confluence, 3T3-L1 preadipocytes (day 0) were stimulated for 48 hours with 520 µmol/L isobutylmethylxanthine, 1 µmol/L dexamethasone, and 167 nmol/L insulin in DMEM/HG supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The preadipocytes were maintained and re-fed every 2 days with DMEM/HG supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 167 nmol/L insulin. To examine the effect of rutin on adipocyte differentiation, the 3T3-L1 preadipocytes received 0, 3, 10, 30, and 100 µM rutin every 2 days starting at 2 days postconfluence until the end of the experiment on day 8.

Oil Red O staining

At day 8 of differentiation, the cells were washed with phosphate-buffered saline, fixed with 10% formaldehyde for 1 hour and then washed with 60% isopropanol. The cells were stained with 0.6% Oil Red O solution (6 parts of 0.6% Oil Red O dye in isopropanol and 4 parts of water) for 15 minutes, washed 3 times with water, and then photographed. Stained Oil Red O was also eluted with 100% isopropanol and quantified by measuring absorbance at 500 nm[10]. A minimum of 3 independent experiments were performed, each in triplicate.

Cell viability with MTT colorimetric assay

The 3T3-L1 preadipocytes were plated into a 96-well microtiter plate at a density of 10^4 cells/well. At 2 days after confluence, the culture medium was replaced by 200 µL of serial dilutions of 0-100 µg/mL rutin, and the cells were incubated for 48 hours. The culture medium was removed and replaced with 20 µL of sterile-filtered L-3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) solution (5 mg/mL phosphate-buffered saline), which was added to each well. The culture was incubated for 2 hours at 37°C, after which the liquid in each well was replaced with 100 µL dimethyl sulfoxide (DMSO). The absorbance of the sample was measured at 570 nm[11].

Adiponectin production

Aliquots of the conditioned medium were taken from 3T3-L1 preadipocytes on day 8. The concentration of adiponectin was assayed using a mouse adiponectin enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn, USA), and the absorbance was measured at 450 nm. Three independent experiments were performed, each in triplicate.

Preparation of total RNA and quantitative reverse transcription polymerase chain reaction

The messenger RNA (mRNA) levels were measured as described by Rhee et al[12]. Total RNA was extracted from 3T3-L1 cells with QIAzol reagent, according to the manufacturer's instructions. A quantity of 1 µg RNA was reverse-transcribed using the oligo (dT) 15 primer and M-MLV reverse transcriptase (Promega, Madison, Wis, USA). After complementary DNA synthesis, the levels of mRNA transcripts were measured by RT-PCR using SYBR Green (Qiagen, Chatsworth, Calif, USA) and Rotor-Gene 3000 (Corbett Research, Sydney, NSW, Australia) using specific primers. The primer sequences were mouse C/EBPα (Fwd: 5'-TGCCTATGAGCACTTCACAA-3', Rev: 5'-AACTCCAGCACCTTCTGTTG-3'), mouse PPARγ (Fwd: 5'-TGGGAACCTGGAAGCTTGTCTC-3', Rev: 5'TGTGGTAAAGGG CTTGATGT-3'), mouse aP2 (Fwd: 5'-GGCTCTGTGCTGCTCCATCT-3', Rev: 5'-GCTGATGATCATGTTGGGCTTG-3'), mouse adiponectin (Fwd: 5'-CTGGAGAAA CCTGCCAAGTA -3', Rev: 5'-AGAGTCGTTGAC GTTATCTGCATAG-3'), and mouse GAPDH (Fwd: 5'-CTGGAGAAACC TGCCAAGTA-3', Rev: 5'-AGTGGGAGTTGC TGTTGAAG-3'). The reaction mixtures were incubated for an initial denaturation at 95°C for 15 minutes, followed by 45 PCR cycles. Each cycle was performed at 95°C for 25 seconds, 57°C for 25 seconds, and 72°C for 25 seconds, respectively. The $\Delta\Delta C_T$ method was used to measure relative quantification, and the levels of transcripts were normalized to GAPDH. The levels of each different mRNA in the vehicle-treated normal cells were designated as 1, and the relative levels of gene transcripts in the other samples were expressed as the fold change. Three independent experiments were performed, each in triplicate.

Statistical analyses

The results are expressed as the means $\pm$ SEM of each group. Analysis of variance followed by Tukey post hoc test was used to test for differences among

the treatment groups. The statistical analyses were performed using the SigmaStat software (Systat Software, San Jose, Calif, USA). The level of significance was uniformly set at $p<0.05$.

Results

Rutin stimulated adipocyte differentiaion in 3T3-L1 cells

Two-day postconfluent 3T3-L1 preadipocytes (day 0) were treated with rutin at 3, 10, 30 and 100 μM every 2 days for 8 days. When preadipocytes differentiated into adipocytes, morphological alterations were observed due to the accumulation of lipid droplets in the cytoplasm. As evidenced by Oil Red O staining, rutin significantly increased lipid accumulation compared to control cells (Fig. 1A). The results from the absorbance measurement of Oil Red O elution (Fig. 1B) showed that 3, 10, 30 and 100 μM rutin increased lipid accumulation as compared to control cells by 120-145% ($p<0.05$). Importantly, rutin (3, 10, 30 and 100 μM) did not affect viability of 3T3-L1 preadipocytes as determined by MTT assay (data not shown).

Rutin enhanced expression of adipogenic transcription factor genes

The authors further examined the alteration of expression of two key transcription factors known to be involved in adipocyte differentiation, C/EBPα and PPARγ. Furthermore, aP2 was also investigated and has been considered a hallmark of adipogenesis. As shown in Fig. 2, the concentrations of rutin at 3, 10, 30 and 100 μM significantly increased the expression of C/EBPα by 1.5, 1.6, 2.2 and 2.1-fold over the basal level, respectively. Rutin at 10, 30 and 100 μM significantly increased the expression of PPARγ by 2.4, 2.8 and 4.0-fold, respectively (Fig. 2). The mRNA levels of aP2 was significantly increased by 1.7 to 2.5-fold during adipocyte differentiation.

Rutin stimulated the gene expression and secretion of adiponectin

The adiponectin concentrations were measured in the medium collected at day 8 of differentiation. The 3T3-L1 cells in normal state released adiponectin by 70±7.4 ng/ml (Fig. 3A). Incubation of preadipocytes with rutin stimulated the secretion of adiponectin as compared to normal adiponectin secretion. The concentrations of rutin at 10, 30 and 100 μM significantly increased adiponectin by 93±0.2, 107±18.0 and 130±13.5 ng/ml, respectively

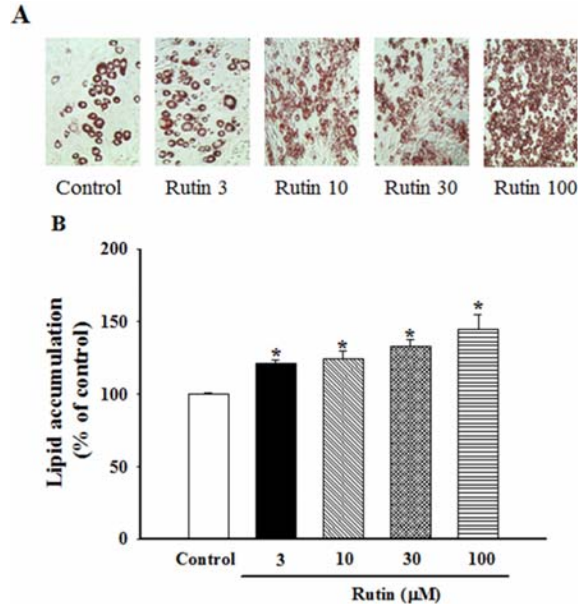

Fig. 1 Effect of rutin on lipid accumulation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes on day 8 of treatment with 3, 10, 30 and 100 mM rutin were stained with Oil Red O (A). Quantification of lipid accumulation was based on the optical density values (at 500 nm) of destained Oil Red O extracted from the adipocytes (B). Values are expressed as mean ± SEM (n = 3). * $p<0.05$ versus control group.

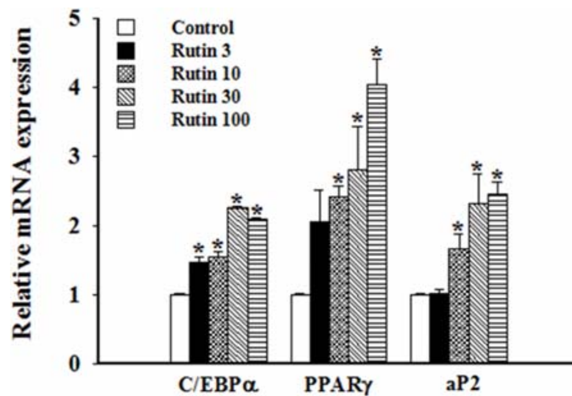

Fig. 2 Effect of 3, 10, 30 and 100 μM rutin on the expression of the adipogenic genes, C/EBPα, PPARγ, and aP2, in 3T3-L1 cells. Values are expressed as mean ± SEM (n = 3). * $p<0.05$ versus control group.

($p<0.05$). Treatment of 3T3-L1 cells with rutin (10, 30 and 100 μM) significantly increased adiponectin mRNA level during adipocyte differentiation by 1.4 to 2.0-fold

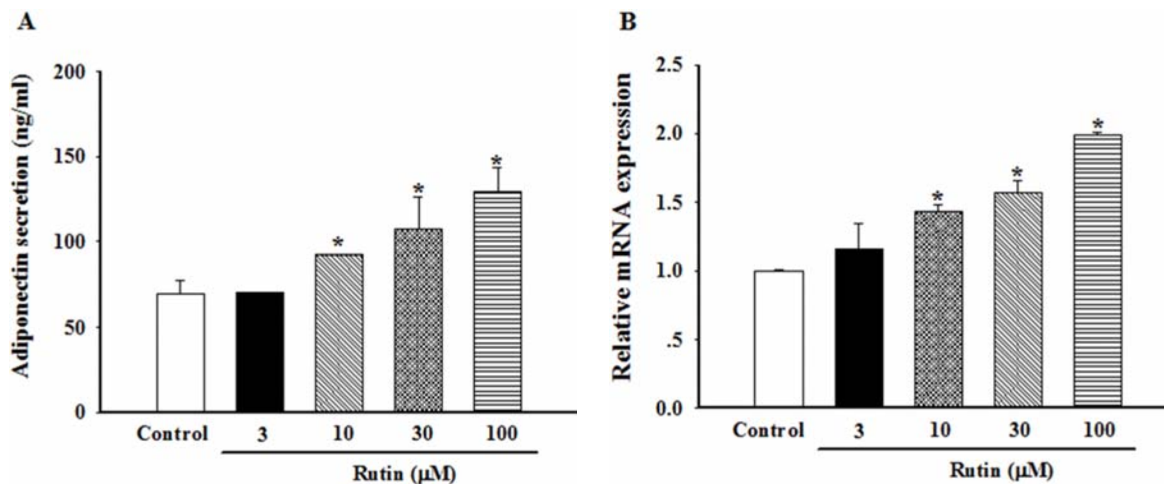

Fig. 3 Effect of rutin on adiponectin secretion (A) and adiponectin mRNA expression (B) in 3T3-L1 cells. Values are expressed as mean ± SEM (n = 3). * $p<0.05$ versus control group.

at day 8 of differentiation (Fig. 3B).

Discussion

In the present study, the authors explored the mechanisms of rutin on adipogenic activity. There has been reported that rutin decreased plasma glucose and increased insulin levels in streptozotocin-induced diabetic rats[13]. The major new findings of the present study are that rutin significantly stimulates lipid accumulation in 3T3-L1 cells and increases two key transcription factors in adipocyte differentiation, C/EBPα and PPARγ. As a result, the expression of aP2 and adiponectin are increased. It has been early documented that C/EBP and PPAR transcription factors work sequentially and cooperatively in stimulating the genetic events leading to differentiation[14]. C/EBPα is reported to have distinct roles in adipose conversion and insulin sensitivity[15].

PPARγ belongs to the nuclear receptor superfamily of ligand-activated transcription factors and is the target of the antidiabetic drug thiazolidinediones (TZDs)[16]. TZDs are known to increase circulating levels of adiponectin by 2 to 3-fold[17,18] and improve insulin resistance by diversion of fat from ectopic sites to subcutaneous adipose tissue[19]. Circulating levels of adiponectin correlate with insulin sensitivity in humans and rodents[20,21] and are reduced in humans with obesity and type 2 diabetes mellitus[22]. Increased expression of adiponectin in a preadipocytic cell line also leads to increased proliferation and differentiation of adipocyte cells. Recent study has shown that the 3T3-L1 adipocytes were treated with 0-250 µM of rutin for 12 and 24 h, inhibited the expression of PPARγ, C/EBPα and leptin, and then up-regulated expression of adiponectin at the protein level[23]. However, in the present study, the authors showed that the preadipocytes were treated with lowered concentrations (3, 10, 30 and 100 µM) of rutin for 8 days, enhanced the lipid accumulation and the expression of PPARγ, C/EBPα, aP2 and adiponectin during the differentiation period. Moreover, it is interesting that the rutin significantly increased the secretion of adiponectin in 3T3-L1 cells.

Our in vitro results strongly support the hypothesis of our study. The results indicate that rutin stimulates the differentiation of 3T3-L1 preadipocytes into adipocytes by enhancing the expression of the adipogenic genes, C/EBPα, PPARγ, and aP2. Moreover, these results suggest that rutin treatment may also improve insulin sensitivity by up-regulating the secretion of adiponectin from 3T3-L1 adipocytes. Thus, the mechanism of the antidiabetic activity of rutin may be similar to the mechanism of TZDs on adipose tissue, especially with regard to the enhancement of PPARγ function. Consequently, it is reasonable to assume that rutin plays a role in reducing insulin resistance.

Conclusion

In conclusion, the authors present the first evidence that rutin stimulates adipogenesis and adiponectin secretion in 3T3-L1 cells. These results also demonstrate the involvement of C/EBPα and PPARγ in rutin-mediated adipogenesis. Stimulation of adipogenic activity and adiponectin secretion may be

one of the antidiabetic mechanisms of rutin. These results support the consumption of rutin as a food supplement for the treatment of diabetes.

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Potential conflicts of interest

None.

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Rutin* กระตุ้นการสร้างเซลล์ไขมันและการหลั่ง *adiponectin* ในเซลล์ไขมันเพาะเลี้ยงชนิด *3T3-L1

จริญญาพร เนาวบุตร, ชุน ฮี จอง, รัน เช

Rutin เป็นสารกลุ่มฟลาโวนอยด์ที่พบในพืชหลายชนิดมีการศึกษาพบว่า rutin มีฤทธิ์ลดระดับน้ำตาลในเลือดและเพิ่มระดับอินซูลินในหนูเบาหวาน สำหรับการศึกษานี้ผู้นิพนธ์มีวัตถุประสงค์เพื่ออธิบายโมเลกุลพื้นฐานของการออกฤทธิ์ต้านเบาหวาน โดยประเมินการออกฤทธิ์ในเซลล์ไขมันเพาะเลี้ยงชนิด 3T3-L1 พบว่า rutin ขนาดความเข้มข้น 3, 10, 30 และ 100 ไมโครโมลาร์ เพิ่มการสะสมไขมันและเพิ่มการแสดงออกของจีน transcription factors ได้แก่ peroxisome proliferator-activated receptor gamma, CCAAT/enhancer-binding protein alpha และ adipocyte fatty acid-binding protein 2 นอกจากนี้ rutin ขนาดความเข้มข้น 10, 30 และ 100 ไมโครโมลาร์ สามารถเพิ่มการแสดงออกของจีน adiponectin ร่วมกับการกระตุ้นการหลั่ง adiponectin ในเซลล์ไขมันที่กำลังเจริญเติบโต ผลการทดลองเหล่านี้บ่งชี้ว่า rutin มีฤทธิ์กระตุ้นการแบ่งตัวของเซลล์ไขมัน โดยผ่านการกระตุ้นการเพิ่มขึ้นของจีน transcription factors และจีนจำเพาะต่อการสร้างเซลล์ไขมันฤทธิ์ของ rutin ในการกระตุ้นการทำงานของ adiponectin และการสร้างเซลล์ไขมันนี้น่าจะเป็นส่วนหนึ่งในการออกฤทธิ์ต้านเบาหวานของการบริโภคอาหารที่มี rutin เป็นองค์ประกอบ