Original Article

Corosolic acid isolation from the leaves of *Eriobotrta japonica* showing the effects on carbohydrate metabolism and differentiation of 3T3-L1 adipocytes

Wei Zong PhD and Guangyuan Zhao PhD

School of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou,China

The extracts of *Eriobotrta japonica* leaves with the 3H-glucose uptaking activity in 3T3-L1 adipocytes were separated by TLC for two times. On the basis of UV-vis spectral, NMR and MS data, corosolic acid was identified as activity components. Moreover, the effects of corosolic acid on carbohydrate metabolism and differentiation of 3T3-L1 adipocytes was studied. The results showed that 3H-glucose uptaking rate in different concentrations of corosolic acid (15μmol/L ,30μmol/L ,and 45μmol/L)group were increased to 108.1%, 112.2% ,118.6%, respectively, compared to control group (without corosolic acid) (p<0.01). Corosolic acid suppressed the differentiation of 3T3-L1 pre-adipocytes and down-regulated the expression of PPAR-γ and C/EBP-α mRNA (p<0.01, vs control group). Corosolic acid promotes the 3H-glucose uptaking, suppresses the differentiation and down-regulates the expression of PPAR-γ and C/EBP-α mRNA in 3T3-L1 adipocytes.

Key Words: *Eriobotrta japonica* leaves, corosolic acid, adipocytes, glucose transport, 3T3-L1 adipocytes

Introduction

Diabetes, particularly obesity and metabolic syndrome related type 2 diabetes (T2D), is a major health problem in the Western world, and it is becoming an increasing threat in developing countries as wealth accumulates and lifestyles change.[1] T2D, which may be caused by metabolic syndrome is characterized by a reduced sensitivity to insulin signaling and a reduced efficiency of glucose transport, primarily in adipocytes and muscle cells, leading to hyperglycemia and hyperinsulinemia.[2-4]

Troglitazone can enhance adipocyte differentiation to increase glucose uptake in cells to decreases blood glucose levels is being widely used for the treatment of T2D.[5,6] However, this could results in excessive accumulation of white adipose tissue. Recent studies showed that white fat tissue accumulation plays crucial role in the development of obesity and T2D.[7] Therefore, there is a contradiction in using troglitazone to treatment of obesity and T2D. Thus, a novel small active molecule with insulin-like actions, but have no effect on accumulation of white adipose tissue is desirable.

Eriobotrya japonica is a subtropical evergreen tree with delicate edible fruits and was considered indigenous to southeastern China.[8] Recent studies showed that the leaves of *Eriobotrta japonica* have many pharmacological effects such as anti-inflammatory activity,[9] antiviral activity.[10] According to recent studies, the triterpene acids (TAL) extracted from *Eriobotrya japonica* leaves were the effective components.[11,12] However, there has been no data on the hypoglycemic components from leaves of *Eriobotrta japonica.* Therefore, it's necessary to investigate the hypoglycemic activity of various organic solvent extracts and identify the major hypoglycemic components from leaves of *Eriobotrta japonica*.

The murine 3T3-L1 preadipocyte is the most early established cell line that has been widely used in the researches of lipid metabolism and adipocyte differentiation.[13]The cell line has also been used as a model for screening small molecules with potential antiobesity properties because it can be differentiated into adipocytes by hormonal treatment.[14]

Therefore, this study was to investigate and identify the major hypoglycemic components from leaves of *Eriobotrta japonica* by a cells model which measurement the insulin mediated glucose uptake in 3T3-L1 adipocytes and to study the effect of the activity components on carbohydrate metabolism and differentiation in3T3-L1 adipocytes.

Materials and methods

Extract of hypoglycemic components from Eriobotrta japonica leaves

A 1000g of sample of *Eriobotrta japonica* leaves (collected in Wuxi, China ,August, 2004) was extracted for 24h with 8000mL methanol at room temperature for three times. The methanol extracts were concentrated under vacuum. The concentrated methanol extracts partitioned successively with petroleum ether, chloroform ($CHCl_3$), acetic ether (EtOAc), and n-hexane. Every fractions were filtered with

Corresponding Author: Professor Wei Zong, School of Food and Biological Engineering, Zhengzhou University of Light Industry, 5 Dongfeng Road, Zhengzhou, Henan, China 450002.
Tel: 86 371 63627115; Fax: 86 371 63556627
Email: zongwei1965@126.com

Whatman No.1 filter paper. The filtrates were evaporated in vacuum and weighted to determine the yield of soluble constituents.

Thin-Layer Chromatography (TLC)

Extracts were spotted in a line on the Silica gel (Haiyang Co., Qingdao, China) TLC plate (10cm×20cm, 1mm) to develop in a developer. In first TLC separation, the developer was a mixture of $CHCl_3$: acetone (4:1, v/v) .In the second TLC separation, developer was a mixture of $CHCl_3$: methanol (95:5 v/v). After being developed, the plate was sprayed with the solution of 10% (v/v) sulfuric acid-alcohol and then heated at 105℃ for 15min, the spots were located by a TLC scanner (CAMAG Ltd., Muttenz, Switzerland) at detection wavelength 520 nm and reference wavelength 700nm. The corresponding fractions were scraped off from the plates according to the R_f values and washed out of Silica gel with methanol.

UV- Vis Spectrometry

UV -Vis Spectrometry absorption spectra of the active components in methanol were recorded on Unico2000 spectrophotometer (Unico, Shanghai, China).

Mass Spectrometry

Mass Spectra of active components were obtained on a Bruker Esquire-LC instrument (Bruker, Germany) for electro-spray (ESI-MS) measurements (solvent: methanol).

Nuclear Magnetic Resonance (NMR) Spectrometry

^1H and ^{13}C-NMR spectra were obtained on a Bruker AMX-500 MHz spectrometer (Bruker, Germany). Spectra were run in DMSO-d6 and referenced to an internal TMS standard.

Cell culture, adipocyte differentiation

Adipocyte differentiation was induced and glucose uptake activity was assessed according to the method of Liu et al [14] with modifications. 3T3-L1 cells (ATCC, Rockville, USA) were grown in DMEM medium (Huamei Co., Shanghai, China) and supplemented with 10% FBS (Huamei Co., Shanghai, China) at 37℃ to confluency in 12-well plates. Adipocyte differentiation was induced by supplementing the media with a combination of 166.7nmol/L insulin (Sigma Co., USA) , 540.5 μ mol/L IBMX (Sigma Co., USA) , and 255.1 nmol /L DEX (Sigma Co., USA) for 48 h followed by insulin alone for an additional 48 h. The media was then replaced with fresh culture medium (DMEM supplemented with 10% FBS) after 2 days and then every three day thereafter. Uptake experiments were performed 11–12 days post induction of differentiation.

Glucose uptake activity assay

Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[3H] glucose (Beijing Atom Technology Co., Beijing, China).[15] Confluent 3T3-L1 adipocytes grown in 12-well plates were washed twice with serum-free DMEM and incubated with 0.5 mL of the same medium at 37℃ for 2 h. The cells were washed three times with Krebs–Ringer–Hepes (KRP) buffer and incubated with 0.9 mL KRP buffer at 37℃ for 30 min. Insulin or extracts were then added and adipocytes were further incubated at 37℃ for 20 min. Glucose uptake was initiated by the addition of 0.1 mL KRP buffer containing 0.037 MBq/L 2-deoxy-D-[^{3}H] glucose and 0.001 mmol/L glucose. After 15 min, the assay was terminated by washing the cells three times with cold PBS. The cells were lysed with 0.7 mL of 1% Triton X-l00 at 37℃ for 20 min. The radio activity retained by the cell lysates was determined by a scintillation counter (Tri-Carb 2100TR, Packard, USA). The effect of samples on glucose uptake increase rate in 3T3-L1 adipocytes was calculated as follow:

$$\text{Glucose uptake increase rate (\%)} = (As/A_0)\times 100\%$$

Here, A_S is the glucose uptake of samples and A_o is the glucose uptake of black control groups.

Oil red O staining

Oil Red O (Huamei Co., Shanghai, China) (0.1 mg/mL) was dissolved in isopropyl alcoho1. Cells were fixed with 10% of formaldehyde for 1 hour at room temperature then stained with 0.1 mg/mL Oil Red O solution for 2 hours at room temperature · All liquid in each well was tipped out and 100 μLof isopropyl alcohol (Huamei Co., Shanghai, China) was added to dissolve the precipitation. The absorbance was determined at 510 nm.[16]

RT-PCR for the expression PPAR-γ and C/EBP-α mRNA

RT-PCR for the expression PPAR-γ and C/EBP-α mRNA was carried out by the modify method previously described.[17] Total RNA from culture 3T3-L1 cells were isolated with TRIzol reagent (Huamei Co., Shanghai, China). 1μg of total RNA was used to produce rRNA using an RT-PCR system (Promega, Madison, WI). The reverse transcription (RT) was performed at 42℃ for 1h and heated at 95℃ for 5 min to inactivate the Avian Myeloblastosis Virus Reserse Transcription (AMV RT) enzyme. The cDNA primers for PPAR-γ (Shanghai Biological Co., Shanghai, China) were forward (5'GACCAC TCGCATTCCT-TT3') and reverse (5'CCACAGACTC GGCACT-CA3'). The cDNA primers for C/EBP-α (Shanghai Biological Co., Shanghai, China) were forward (5'GAGGAGGACGAGGCGAAGCA3') and reverse (5' TCCAGCCACCCGAAACCA3'). The cDNA primers for β-actin were forward (5'GTCTTTACGGATG-TCAA CG3') and reverse (5'CCCTGTATGCCTCTGGTC3'). The reaction were denatured at 94℃ for 1 min, annealed at 57℃ for 1 min, and extended at 74℃ for 2 min. Amplication was carried out using 33cycles and final extension was performed at 72℃ for 7 min. The PCR products were loaded onto 1.5% agarose gel stained with ethidium bromide. The relative level of RT-PCR reaction products was measured with Alpha Innotech Imager (spot densitometry program, San Leandro, CA) under UV light. β-actin expression was used to normalize relative level of the expression PPAR-γ and C/EBP-α mRNA.

Statistical analysis

Data were expressed as the means±standard error of the means (S.E.M.). Statistical analyses were performed using a computer program provided in SAS.[18] Differences among groups were considered to be statistically signifi-

cant when the $p<0.05$.

Results and discussion

Hypoglycemic effects of different part from the leaves of Eriobotrta japonica

The yields of methanol extracts and the partitioned fractions from 1000.0g of freezed-dried *Eriobotrta japonica* leaves are shown in table1. The results indicate that the yield of methanol extracts was 220.52g. The partitioned fractions of methanol extracts increases with increasing polarity of solvent.

Comparison of hypoglycemic effects of different part from the leaves of *Eriobotrta japonica* by a cells model which measurement the insulin mediated glucose uptake in 3T3-L1 adipocytes is shown in Figure 1.

Figure 1 shows that hypoglycemic effects of different part from the leaves of *Eriobotrta japonica*. Methanol extracts of *Eriobotrta japonica* leaves exhibited strong hypoglycemic activity. Furthermore, the n-BuOH extracts (BE) fraction of methanol extracts exhibited stronger hypoglycemic activity than others fractions.

Table1. Yields of differ ent organic solvent extracts from the leaves of *Eriobotrta japonica*

solvent	yield (g)	solvent	yield (g)
methanol	220.52±0.11	EtOAc	32.23±0.04
petroleum ether	7.14±0.02	n-BuOH	81.26±0.06
$CHCl_3$	16.67±0.02		

Based on 1000.0g of freezed-dried *Eriobotrta japonica* leaves for different organic solvent. Value are means±standard deviation of three replicate analyses.

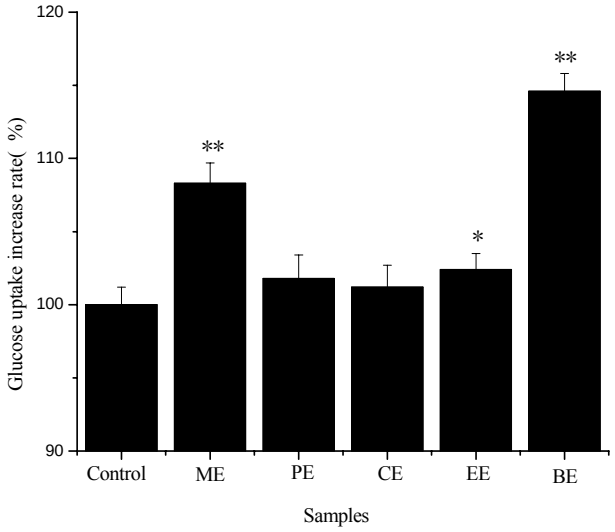

Figure 1. The effect of the extract of *Eriobotrta japonica* leaves for different organic solvent on glucose uptake in 3T3-L1 adipocytes in the absence and presence of extract (20mg/ml). Adipocytes in 12-well plates were incubated for 20 min without treatment, or with the extract of *Eriobotrta japonica* leaves for different organic solvent, then assayed for 2-deoxy-d-[^{3}H] glucose uptake. Data are means±S.E.M., $n = 6$. * $p<0.05$, ** $p< 0.01$, vs. control.

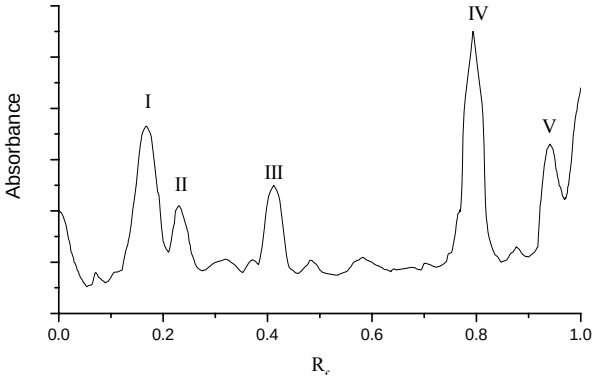

Figure 2. TLC profile of HE from the leaves of *Eriobotrta japonica* as observed by TLC scanner (detection wavelength 520, reference wavelength 700nm). Solvent system: $CHCl_3$/acetone (4:1, v/v). After developed, the plate was partial sprayed with the solution of 10% (v/v) sulfuric acid-alcohol and then heated at 105℃ for 15min

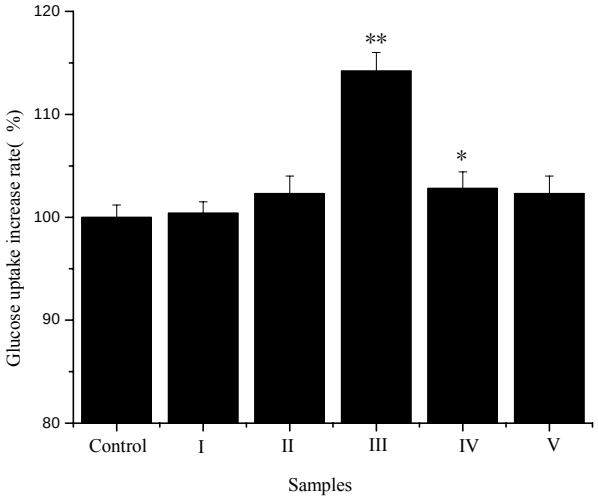

Figure 3. The effect of the fractions of BE on glucose uptake in 3T3-L1 adipocytes in the absence and presence of fractions (20mg/mL). Adipocytes in 12-well plates were incubated for 20 min without treatment, or with the the fractions of BE, then assayed for 2-deoxy-d-[^{3}H] glucose uptake. Data are means±S.E.M., $n = 6$. * $p<0.05$, ** $p < 0.01$,vs. control.

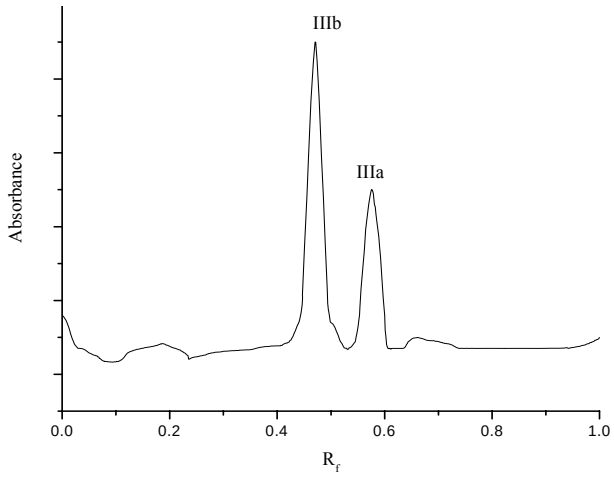

Figure 4. TLC profile of fraction III from the leaves of *Eriobotrta japonica* as observed by TLC scanner (detection wavelength 520, reference wavelength 700nm) . Solvent system: $CHCl_3$/methanol (95:5 v/v). After developed, the plate was partial sprayed with the solution of 10% (v/v) sulfuric acid-alcohol and then heated at 105℃ for 15min

Hypoglycemic effects of different fraction from HE

With the Solvent system ($CHCl_3$: acetone =4:1, v/v), the BE fraction of methanol extracts was separated into five fractions by TLC (Fig 2). The R_f of five fractions were 0.16, 0.23, 0.42, 0.79, and 0.94, respectively. Comparison of hypoglycemic effects of five fraction, fraction Ⅲ (R_f =0.42) possessed remarkable hypoglycemic activities (Fig 3).

Hypoglycemic effects of different fraction from fraction Ⅲ

The active fraction Ⅲ was further purifed on a silica gel plate by using a $CHCl_3$/methanol (95:5 v/v) solvent system. Two subfractions of fractions Ⅲ separated by TLC are shown in Figure 4. The R_f of the two subfractions were 0.47 and 0.58. Comparison of hypoglycemic effects of subfractions Ⅲa and subfractions Ⅲb, subfractions Ⅲa (R_f =0.58) possessed remarkable hypoglycemic activities (Fig 5).

Structure of active components

The existence of markedly strong hypoglycemic activity were observed in subfraction Ⅲa. Therefore, the present investigation of purification and identification was focused more on this subfraction. Subfraction Ⅲa was further developed on a silica gel plate by using various solvent systems, but no seperation was obtained. So the Structure data of this active components are determined. The structure data were as follows:

White power (methanol). mp251~254℃. The λmax in MeOH solution was 206nm. ESI^--MS (m/z): 471[M-1], ESI^+-MS (m/z): 495[M+Na]; ^{1}H-NMR(pyridine-d5,500MHz)δ: 0.97(3H,d,J=6.5Hz), 1.01(3H, d, J=6.5Hz), 0.94, 1.03, 1.06, 1.20,1.25 (3H×5, s,), 2.61(1H, d, J=11.0Hz, H-18), 3.38(1H, d, J=9.5Hz, H-3β), 4.08 (1H, td, J=11.0, 4.5Hz, H-2β) ,5.46 (1H, t-like, J=3.5Hz, H-12); ^{13}C-NMRδ: 48.1 (C-1), 68.7 (C-2), 83.9 (C-3), 40.1 (C-4), 55.8 (C-5), 18.9 (C-6), 33.7(C-7), 40.2(C-8), 47.8(C-9), 37.3 (C-10), 24.1(C-11), 128.19(C-12), 140.1(C-13), 42.4(C-14), 29.5(C-15), 26.3 (C-16), 48.3(C-17), 54.4 (C-18), 72.4(C-19), 42.2 (C-20), 27.1(C-21), 38.5 (C-22), 29.6 (C-23), 22.2 (C-24), 16.8 (C-25), 17.5(C-26), 24.6 (C-27), 180.7(C-28), 27.3(C-29), 16.6 (C-30).

These data agreed with that of corosolic acid (CA) described by Yamagishi et al.[19] Thus, the molecular of $C_{30}H_{48}O_4$ was suggested, and the structural formula was shown in Figure 6.

Effect of CA on glucose transport in 3T3-Ll adipocytes

When the 3T3-Ll adipocytes were incubated in a medium containing 2-deoxy-d-[3H] glucose, although to a lesser extent than 1.0nmol/L insulin, the CA stimulated glucose uptake rate in 3T3-Ll adipocytes significantly. The results showed that 3H-glucose uptaking rate in different concentrations of CA (15μmol/L, 30μmol/L, and 45μmol/L) groups were increased to 108.1%, 112.2% ,118.6%, respectively, compare to control group (without CA) (p<0.01). (Fig 7)

To test whether CA could further potentiate insulin's glucose uptake activity, 30μmol/L CA was added to insulin at various concentrations (0–20 nmol/L). Glucose uptake was augmented from that of insulin alone (Fig 8),

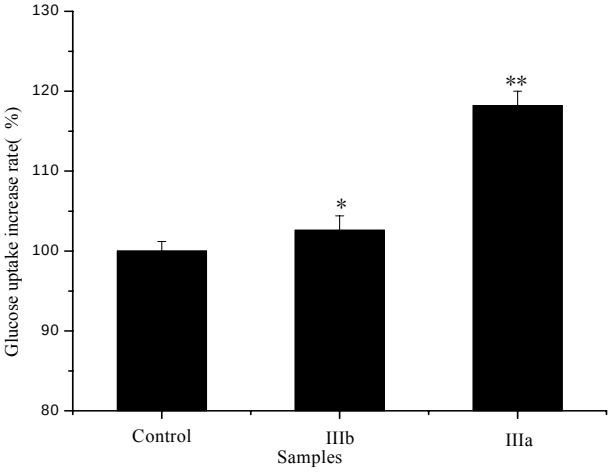

Figure 5. The effect of the subfractions of Ⅲ on glucose uptake in 3T3-L1 adipocytes in the absence and presence of subfractions (20mg/mL). Adipocytes in 12-well plates were incubated for 20 min without treatment, or with the the subfractions of Ⅲ, then assayed for 2-deoxy-d-[^{3}H] glucose uptake. Data are means±S.E.M., n = 6. * p <0.05, ** p < 0.01, vs. control.

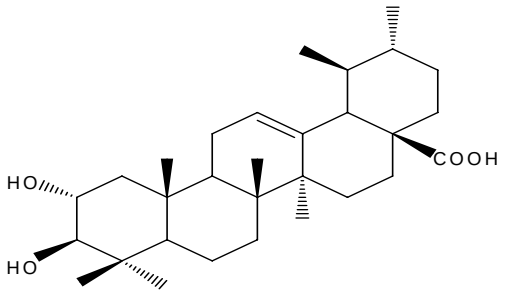

Figure 6. Structures of CA

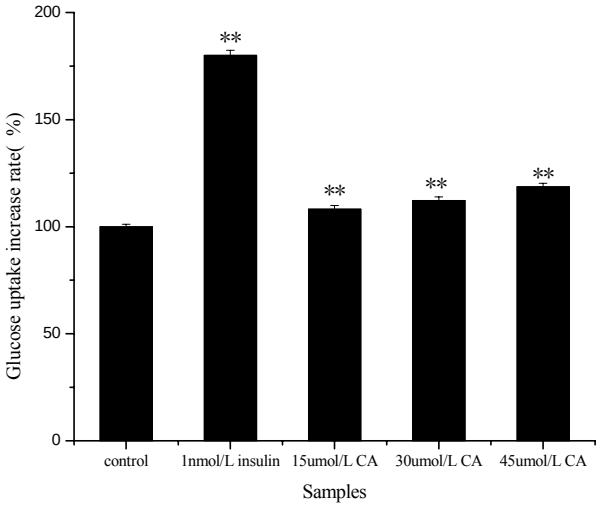

Figure 7. The effect of CA on glucose uptake in 3T3-L1 adipocytes in the absence and presence of insulin (1 nmol/L). Adipocytes in 24-well plates were incubated without treatment, or with insulin, or with CA, then assayed for 2-deoxy-d-[3H] glucose uptake. Data are means±S.E.M., n = 6. ** p < 0.01,vs. control.

indicating that synergistic effect exists between CA and insulin.

Effect of CA on fat content changes in 3T3-Ll adipocytes

The effect of CA on fat content changes in 3T3-Ll adipocytes was shown in Figure 9.The fat contents in the differentiating 3T3-L1 cells decreased with the increase of

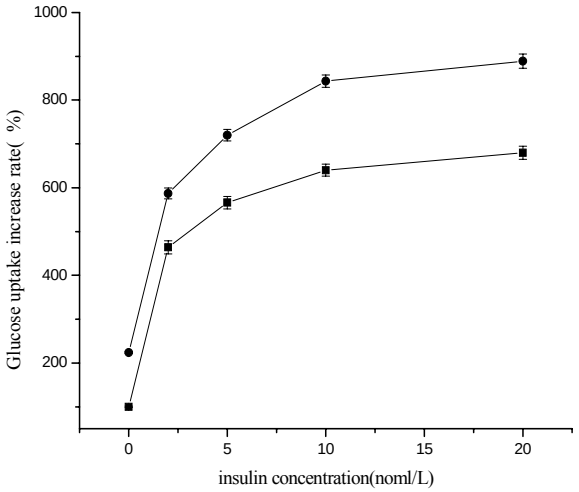

Figure 8. Combined effects of insulin and CA on glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells were incubated with insulin in the presence or absence of 30μmol/LCA, and then assayed for the glucose uptake activities. Data are means±S.E.M., $n = 6$. ■,insulin; ●,insulin +30umol/L CA.

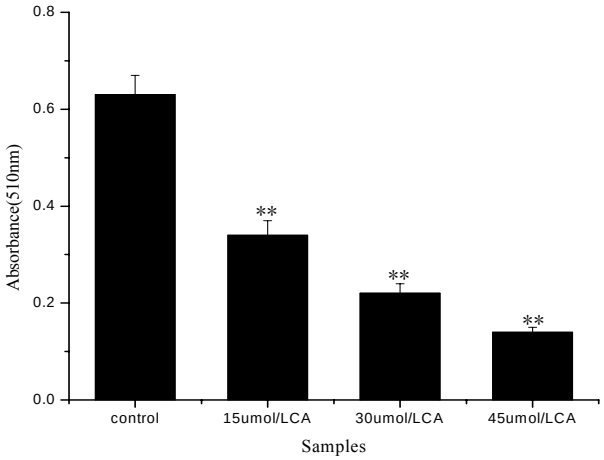

Figure 9. The effect of CA on the fat contents in 3T3-L1 adipocytes in the absence and presence of different concentration CA (15μmol/L, 30μmol/L, and 45μmol/L). Adipocytes in 24-well plates were incubated without treatment, or with with CA, then assayed for fat contents by oil red O staining. Data are means±S.E.M., $n = 6$. ** $p < 0.01$, vs. control.

CA concentrations in medium. This demonstrated that CA has a dose-dependent inhibition effect on 3T3-L1 cells' differentiation.

Effect of CA on the expression of PPAR-γ and C/EBP-α mRNA

The Effect of CA on the expression of PPAR-γ and C/EBP-α mRNA in 3T3-L1 adipocytes were determined using RT-PCR (Fig 10 and Fig 11). The RT-PCR analysis showed that the expression of PPAR-γ and C/EBP-α mRNA in 3T3-L1 adipocytes decreased with the increase of CA concentrations in medium. This demonstrated that CA also has a dose-dependent inhibition effect on the expression of PPAR-γ and C/EBP-α mRNA in 3T3-L1 adipocytes.

Glucose and fat are two major substrates for energy production in animals. Coordination between their metabolism in providing energy is sophisticated and is regulated by many hormonal and metabolic factors.

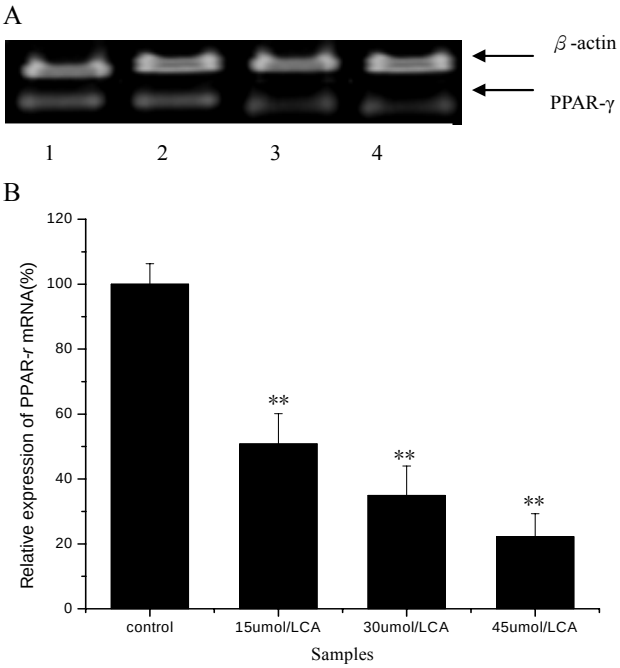

Figure 10. The effect of CA on the Expression of PPAR-γ mRNA in 3T3-L1 adipocytes in the absence and presence of different concentration CA (15μmol/L ,30μmol/L ,and 45μmol/L).(A) The expression of PPAR-γ mRNA .Lane 1:control; Lane 2: 15μmol/L CA; Lane 3: 30μmol/L CA; Lane 4: 45μmol/L CA. (B) The analysis of PPAR-γ mRNA from RT-PCR. Spot densitometry program of Alpha Innotech Imager (San Leandro, CA) was used to measure the relative level of RT-PCR reaction products under UV light. The relative level of PPAR-γ mRNA was expressed as a percentage of PPAR-γ vs β-actin mRNA levels. Data are means±S.E.M., $n = 6$. ** $p < 0.01$, vs. control.

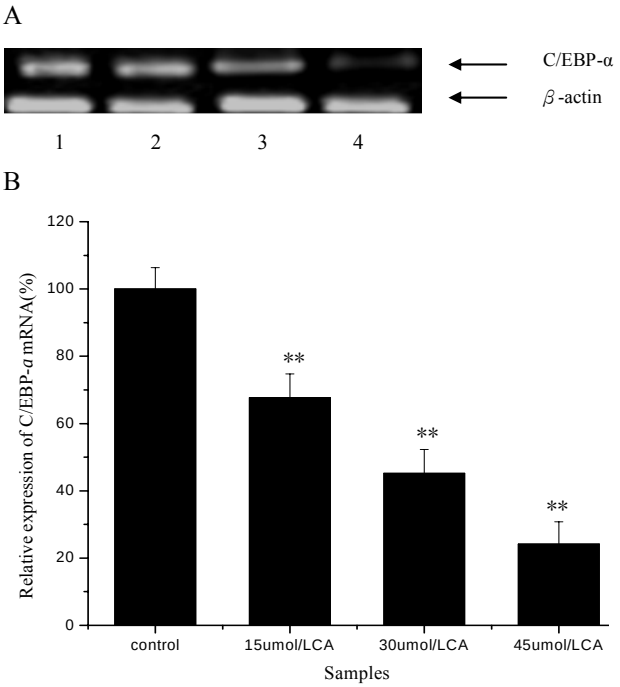

Figure 11. The effect of CA on the Expression of C/EBP-αmRNA in 3T3-L1 adipocytes in the absence and presence of different concentration CA (15μmol/L ,30μmol/L ,and 45μmol/L).(A) The expression of C/EBP-α mRNA .Lane 1:control; Lane 2: 15μmol/L CA; Lane 3: 30μmol/L CA; Lane 4: 45μmol/L CA. (B) The analysis of C/EBP-α mRNA from RT-PCR. Spot densitometry program of Alpha Innotech Imager (San Leandro, CA) was used to measure the relative level of RT-PCR reaction products under UV light. The relative level of C/EBP-α mRNA was expressed as a percentage of C/EBP-α vs β-actin mRNA levels. Data are means±S.E.M., $n = 6$. ** $p < 0.01$, vs. control.

Disturbance of the energy homeostasis may cause serious clinical syndromes that are manifested by abnormal blood glucose or fatty acid levels. For instance, level is increased. Although adipose tissue accounts for only 5-20% of glucose disposal, much of the work on insulin-stimulated glucose transport has been performed in adipocytes, due to the fact that many mechanistic studies with regard to insulin's action have been easier to carry out in this tissue.

Carbohydrate metabolism and differentiation of 3T3-L1 adipocytes are associate with diabetes.[20] peroxisome proliferator-activated receptor (PPAR)-γ and the CCAAT/enhancer binding Protein C/EBP family (C/EBP-α,β,andδ) were critical factors in 3T3-L1preadipocyte differentiation.[21] PPARγ is a member of the nuclear receptor superfamily of transcription factors and it is predominatly expressed in adipose tissue.[22] C/EBP family are basic leucine zipper transcription factors. [23,24] C/EBP family and PPARγ are sequentially expressed during 3T3-L1 preadipocyte differentiation.[25-28] The promoters of several adipogenic genes are regulated by these transcription factors. Therefore, PPARγ and C/EBP family are key transcription factors for adipocytes differenation.[29] In this study, the expression of PPARγ and C/EBP-α induced by DEX, IBMX, and insulin was inhibited together with adipogenis measure by oil red O staining. So it is appears that CA decreased fat accumulation by inhibited the expression of PPARγ and C/EBP-α. We therefore suggest that its effect on glucose uptake might be one of the mechanisms for its anti-diabetic action.

In this work we found that CAwhich separated from leaves of *Eriobotrta japonica* has stimulates glucose uptake and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Furthermore, CA not only stimulates glucose transport, but also inhibits the differentiation of preadipocytes into adipocytes. This suggests that, unlike most other anti-diabetic drugs, CA may reduce blood glucose without increasing adiposity

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