Ginsenoside Rb1 promotes browning through regulation of PPARγ in 3T3-L1 adipocytes

Qianqian Mu a,1, Xin Fang a,1, Xiaoke Li a, Dandan Zhao b, Fangfang Mo b, Guangjian Jiang b, Na Yu a, Yi Zhang a, Yubo Guo a, Min Fu c, Jun-Li Liu c, Dongwei Zhang b, Sihua Gao b, *

a Preclinical Medicine School, Beijing University of Chinese Medicine, Beijing 100029, China
b Diabetes Research Center, Beijing University of Chinese Medicine, Beijing 100029, China
c The Research Institute of McGill University Health Center, Montreal, Quebec H4A 3J1, Canada

ARTICLE INFO
Article history:
Received 10 September 2015
Accepted 11 September 2015
Available online 14 September 2015

Keywords:
Ginsenoside Rb1
PPARγ
Browning
Mitochondrial respiration
3T3-L1 adipocytes

ABSTRACT
Browning of white adipocyte tissue (WAT) has received considerable attention due to its potential implication in preventing obesity and related comorbidities. Ginsenoside Rb1 is reported to improve glycolipid metabolism and reduce body weight in obese animals. However whether the body reducing effect mediates by browning effect remains unclear. For this purpose, 3T3-L1 adipocytes were used to study the effect of ginsenoside Rb1 on browning adipocytes specific genes and oxygen consumptions. The results demonstrate that 10 μM of ginsenoside Rb1 increases basal glucose uptake and promoted browning evidenced by significant increases in mRNA expressions of UCP-1, PGC-1α and PRDM16 in 3T3-L1 mature adipocytes. Further, ginsenoside Rb1 also increases PPARγ activity. And the browning effect is abrogated by GW9692, a PPARγ antagonist. In addition, ginsenoside Rb1 increases basal respiration rate, ATP production and uncoupling capacity in 3T3-L1 adipocytes. Those effects are also blunted by GW9692. The results suggest that ginsenoside Rb1 promote browning of 3T3-L1 adipocytes through induction of PPARγ. Our finding offer a new source to discover browning agonists and also useful to understand and extend the applications of ginseng and its constituents.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Browning of white adipocyte tissue (WAT) has gained interest in recent years owing to potential role in slimming of obesity [1] and reducing insulin resistance [2]. Inducing uncoupling protein 1 (UCP-1) mRNA expression in WAT benefits for browning and increasing mitochondrial density and respiratory capacity [3]. Increased expression of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α) and UCP-1 content further contributes to mitochondrial biogenesis and browning in subcutaneous inguinal in rats [4]. In the meanwhile, browning of WAT in mice is also marked by increasing expression of K1F1-R1F1-Z1 homologous domain containing 16 (PRDM16) which was responsible for development of brown adipocyte tissue [5]. The increased expression of browning specific genes in WAT benefits for glucose tolerance and energy expenditure in obesity resistance treatment [6].

Activation of peroxisome proliferator activated receptor gamma (PPARγ) signalling directly drives UCP-1 expression and contributes to browning of WAT in animals and human [7]. Activated PPARγ in adipose tissue guarantees a healthy secretion of adipocytokines which contributed to insulin action in peripheral tissues [8]. However, some of PPARγ agonists may have the potential to increase heart attack incidences and other health problems [8 11]. Interestingly, some of PPARγ agonists that originated from natural products have been demonstrated to exhibit significant effect on improving metabolic parameters with no obvious side effects in diabetic animal models [12]. Therefore, traditionally used medicinal plants may offer a new promising source for PPARγ agonists. Ginsenoside Rb1 is one of main active ingredients originated from one of traditional used herbs named Panax ginseng C. A. Meyer. Ginsenoside Rb1 has been reported to reduce body weight gain and hepatic fat accumulation, and improve glucose tolerance.
in high fat diet (HFD) induced obesity rats [13,14] and mice [15]. Further, ginsenoside Rb1 attenuates central inflammation and leptin resistance [16] and reduces the release of free fatty acid and alleviates the ectopic deposit of triglyceride by up-regulating the expression of perilipin in adipose tissue [17,18]. In addition, ginsenoside Rb1 shows binding affinity to PPARγ which contributed to induce adipogenesis [19]. Moreover, ginsenoside Rb1 protects cardiomyocytes with no observed adverse effects in men and non-pregnancy women [20–22]. These prompt us to speculate that ginsenoside Rb1 may have beneficial health effects on browning of WAT. To test the hypothesis, here we investigate the possible effect of ginsenoside Rb1 on UCP-1, PGC1α and PRDM16 expression, and its possible mechanisms in 3T3-L1 adipocytes.

2. Material and methods

2.1. Reagents

Ginsenoside Rb1 was bought from China national institute for food and drug control (Purity≥98%; Beijing, China) and prepared as 10 mM stock solution in sterile distilled water. GW9662 was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), and prepared as 50 mM stock solution in dimethyl sulfoxide (DMSO). The rest of chemicals and cell culture additions, except individually specified, were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

3T3-L1 preadipocytes were obtained from Peking Union Cell Source Center and cultured in DMEM supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 1% gentamicin, 0.05% biotin. The medium was changed every 2–3 days. Then the confluent cells were cultured in DMEM medium supplemented with 10% FBS, 1 μM insulin, 0.25 μM dexamethasone, and 0.25 mM isobutylmethylxanthine for 2 days. Further, the cells were incubated with 10% FBS in DMEM in the presence of 100 nM insulin for 2 days. Then the medium was changed to DMEM supplemented with 10% FBS in DMEM for another 3–5 days. Differentiated cells were used for further analysis.

2.3. Glucose uptake assay

The differentiated 3T3-L1 adipocytes were starved in DMEM medium with 0.1% gentamicin, 0.05% biotin in the presence of palmitate (0.5 mM) or 0.5% BSA overnight. Then the cells were gently washed twice with 1 × KRPH buffer (20 mM HEPES, 5 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, pH 7.4) and preincubated in glucose uptake buffer (118 mM NaCl, 6 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 12.5 mM HEPES) with ginsenoside Rb1 for 40 min. After the cells were stimulated with or without insulin (100 nM) and ginsenoside Rb1 for 20 min in glucose uptake buffer, 0-glucose/2-deoxy-glucose (2-DG) mix solution were added to the medium (ratio is 50 μl/0.5 ml). Then all the solution was removed, and the cells were lysed with lysis buffer. A certain amount of assay mixture was added to each well and the plate was incubated at RT for 30–120 min. The plate were monitored the absorbance ratio increase at 570/610 nm with an absorbance plate reader. For negative controls, the cells were untreated with insulin, ginsenoside Rb1 and 2-DG.

2.4. RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was extracted using Tripure (Roche; Indianapolis, USA) according to the manufacturer’s protocol. In brief, a certain amount of Tripure was used to lysis the cells. Then the cell lysate was transferred to the tube and incubated for 5 min at RT. Further, each sample was spinning at 12,000 × g for 15 min at 4 °C followed by adding chloroform. The separated upper phase was placed in a new tube and RNA was harvested by isopropanol precipitation.

To generate cDNA for qPCR, reverse transcriptase kit was used. Briefly, 5 μg of total RNA, 0.5 μg of Oligo (dT) 18 primer, 0.2 μg of random hexamer primer, 4 μl of 5X reaction buffer for reverse transcriptase, 0.5 μl of RiboLock™ RNase inhibitor, 2 μl of dNTP Mix and 1 μl of RevertAid™ H minus reverse transcriptase (200 μl) were added, and incubated at 42 °C for 1 h. Then the reaction was terminated at 70 °C for 10 min.

The resultant cDNA was diluted to 100 μl and 4 μl were used for each 35 μl reaction containing 1.25 U of Taq DNA polymerase (Applied Biosystems; Grand Island, NY, USA), 1x reaction buffer, 1.5 mM MgCl2, 0.5 mM dNTP, 0.4x SYBR green I dye (Molecular Probes; Grand Island, NY, USA), and 357 nM each primer. The cDNA products were denatured at 95 °C for 10 min, followed by 35 PCR cycles (denaturation: 95 °C for 30 s, annealing: 58 °C for 1 min, extension: 72 °C for 1 min). qPCR was performed in the 7500 Real-Time PCR System (Applied Biosystems) and analyzed by using the Ct method (7500 System SDS Software). qPCR primers sequences for UCP-1, PGC-1α and PRDM16 were described as previous published [23]. β-actin was used as an internal control.

2.5. PPAR response element (PPRE) luciferase gene assay

COS-1 cells (ATCC No. CRL-1650) were obtained from Peking Union Cell Source Center and cotransfected in batch by adding 4.5 μg full-length murine PPARγ2-pSV Sport, with 4.5 μg 3 × multimerized PPRE-luciferase reporter and 27 μl X-treme Gene 9 transfection reagent in serum-free Opti-mem reduced serum media (Gibco). After 18 h incubation at 37 °C in a 5% CO2 incubator, transfected cells were plated in triplicate in white 96-well plates at a density of 100,000 cells per well. After replating, cells were treated with either ginsenoside Rb1 or control only. After 18 h incubation, treated cells were developed with brite lite plus (Perkin Elmer; Waltham, Massachusetts, USA) and read in 96-well luminescence Perkin Elmer EnVision multilabel plate reader. Graphs were plotted in triplicate as fold change of treated cells over DMSO-treated control cells.

2.6. Oxygen consumption measurements

Oxygen consumption rate (OCR) measurements were performed using a Seahorse Biosciences XF Extracellular Flux Analyzer (XF Analyzer). 3T3-L1 preadipocytes were plated in quintuple at a density of 30,000 cells per well in XF analyzer microplates (Seahorse Biosciences; Massachusetts, USA) coated with 0.2% gelatin. Then the differentiated cells were treated with either ginsenoside Rb1 or control only. After 48 h incubation, the growth medium were exchanged for XF basic medium (Seahorse Biosciences) supplemented with 11 mM glucose, 2 mM glutamine and 2 mM pyruvate. OCR measurements were 5 min periods following 3 min mix periods. Adipocytes were incubated with sequential addition of 0.5 μM oligomycin, 5 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and 1 μM rotenone with 0.1 μM antimycin A (MP Biomedicals; Santa Ana, CA, USA).

2.7. Statistics

Data were presented as the mean ± SEM. A p value of < 0.05 was considered statistically significant. All the experiments were carried out in triplicate or quadruplicate. Differences between two groups were determined using student’s t-test. Differences among
more than two groups were evaluated using ANOVA (two-way; GraphPad Prism version 6) with Dunnett’s post hoc test.

3. Results

1 Exposure of 3T3-L1 mature adipocytes to ginsenoside Rb1 increased basal glucose uptake

Browning of WAT will increase glucose uptake and contribute to energy expenditure [1]. First, we investigated whether ginsenoside Rb1 treatment will increase glucose uptake in 3T3-L1 mature adipocytes. The differentiated 3T3-L1 adipocytes were incubated with various concentrations of ginsenoside Rb1 (0.01–100 μM) for 1 h. The results showed a dose-dependently increased basal glucose uptake in the presence of ginsenoside Rb1 (0.1–100 μM) in 3T3-L1 mature adipocytes (Fig. 1A). Ginsenoside Rb1 exhibited maximal effect on glucose uptake at 10 μM (167%, p < 0.05). Subsequently, 10 μM of ginsenoside Rb1 was used to study insulin (100 nM) stimulated glucose uptake in 3T3-L1 adipocytes. Interestingly, ginsenoside Rb1 increased glucose uptake by 110% but the increase did not reach statistical difference (Fig. 1B, p = 0.0541). The results indicate that ginsenoside Rb1 treatment increases basal glucose uptake and has a trend to increase insulin stimulated glucose uptake in 3T3-L1 mature adipocytes.

2 Ginsenoside Rb1 induces increased expression browning specific genes in 3T3-L1 differentiated adipocytes

To investigate whether ginsenoside Rb1 could induce the expression of browning adipocyte-specific genes in WAT, 3T3-L1 differentiated adipocytes were treated with 10 μM of ginsenoside Rb1 for 24 h. Then the total RNA was extracted from the harvested cells. The browning specific genes were evaluated by real time PCR. As shown in Fig. 2, ginsenoside Rb1 treatment significantly increased mRNA expressions of UCP-1, PGCG1-z and PRDM16 in 3T3-L1 differentiated adipocytes by 1.8, 1.3 and 1.9 fold, respectively (p < 0.05). These results suggest ginsenoside Rb1 treatment triggers upregulation of browning specific genes and promotes browning in 3T3-L1 adipocytes.

3 Ginsenoside Rb1 promotes browning is through PPARγ dependent manner

PPARγ has been reported as a master regulator of white adipocyte browning and insulin sensitizer [24,25]. We speculate that ginsenoside Rb1 induced browning was through regulation of PPARγ. Cos-1 cells transfected PPRE-luciferase reporter were incubated with 10 μM of ginsenoside Rb1 for 18 h. As shown in Fig. 3, ginsenoside Rb1 dramatically increased PPARγ activity. Further, when GW9662 (a PPARγ inhibitor) was administrated to the media, the upregulation of ginsenoside Rb1 on PPARγ activity was markedly abrogated, which is almost close to the activity inhibited by GW9662 alone. In addition, the upregulation of browning genes resulted from ginsenoside Rb1 treatment were also significantly reduced to control level after inclusion of GW9662 (Fig. 2A–C). The results indicate that ginsenoside Rb1 induces browning specific genes is through a specific induction of PPARγ in 3T3-L1 mature adipocytes.

4 Ginsenoside Rb1 significantly increased mitochondria respiration and energy metabolism

To further explore the browning effect of ginsenoside Rb1 on mitochondrial respiratory function in WAT, we next evaluate OCR alterations using an extracellular flux analyzer XF Analyzer (Seahorse Bioscience) in 3T3-L1 differentiated adipocytes. As shown in Fig. 4A, the OCR of ginsenoside Rb1 treated adipocytes remained higher than the untreated adipocytes (control). First, basal mitochondrial respiration of ginsenoside Rb1 treated cells was increased by 2.4 folds compared to control (p < 0.05). Second, to further clarify the ATP production in mature adipocytes, oligomycin was employed to inhibit the ATP synthase. As shown in Fig. 4C, ATP production of cells in ginsenoside Rb1 treatment was increased up to 3.5 folds compared to control (p < 0.05). The differences reflected ginsenoside Rb1 has the capacity to upregulate ATP turnover. Third, treatment of adipocytes with ginsenoside Rb1 didn’t change the maximal mitochondrial respiratory capacity in comparison with control by addition of the electron transport chain decoupler FCCP (Fig. 4D). Fourth, uncoupling effect of ginsenoside Rb1 treated adipocytes was significantly increased by 2.6 folds compared to control (Fig. 4E). In addition, when GW9692 was administrated to adipocytes with ginsenoside Rb1, the effects of ginsenoside on basal respiration, ATP production, maximal respiration and uncoupling capacity of adipocytes were significantly attenuated. Taken together, these data indicate that 10 μM of ginsenoside Rb1 significantly improved mature adipocytes mitochondrial respiratory effects as shown by increasing basal mitochondrial respiratory, ATP production and uncoupling capacity. And the effect of ginsenoside Rb1 on adipocytes mitochondrial respiratory capacity was through induction of PPARγ.

![Fig. 1](image-url)

Fig. 1. The effect of ginsenoside Rb1 on basal and insulin-mediated glucose uptake in 3T3-L1 mature adipocytes. (A) Basal glucose uptake of ginsenoside Rb1 treatment. (B) Insulin stimulated glucose uptake of ginsenoside Rb1 treatment. Data represent mean ± SEM of four independent experiments. *p < 0.05 was considered significant.
In the present study, we found that ginsenoside Rb1 increased basal glucose uptake. Further, ginsenoside Rb1 promoted browning through upregulation of PPARγ activity via increasing mRNA expressions of UCP1, PGC-1α and PRDM16. The browning effect was also reflected on mitochondria respiration and energy metabolism evidenced by increasing basal respiration, ATP production and uncoupling capacity in 3T3-L1 adipocytes.

Browning of WAT promotes glucose uptake in adipose tissue. Our study found that 10 μM of ginsenoside Rb1 treatment increased basal glucose uptake and also has a trend to increase insulin stimulated glucose uptake in 3T3-L1 adipocytes. However, Shang et al. [26] claimed that 1 μM of ginsenoside Rb1 treatment increased both basal and insulin stimulated glucose uptake. The inconsistency between two studies are possible owing to dosage and duration of treatment of ginsenoside Rb1. Insulin signals triggers glucose consuming and thermogenesis in classical BAT [27]. However, it was reported that lack of insulin may not blunt browning of WAT in primary pre-adipocytes [28]. This is in line with our current finding that ginsenoside Rb1 did not increase insulin stimulate glucose uptake but did increase basal insulin uptake. Ginsenoside Rb1 was reported to have the potential to activate AMP-activated protein kinase, stimulated fatty acid oxidative enzymes expression, and suppressed lipogenesis, so we imagined that ginsenoside Rb1[14] induced browning may be not strictly insulin dependent, and some other stimulators may be involved in this respect.

Emerging evidence demonstrates that PPARγ is a strong inducer of browning of rodent and human adipocytes [29]. Activation of PPARγ increases insulin secretion and sensitivity that contributed to obesity/insulin resistance treatment [30]. PPARγ agonists triggered browning in white adipocytes marked by rising PRDM16 and UCP-1 expression [31]. PGC-1α/bounds to PPAR-γ and coactivates PPAR-γ to stimulate browning of WAT, which contributed to mitochondrial biogenesis and respiratory function [32,33] which facilitated energy expenditure. Previous study found that ginsenoside Rb1 stimulated adipogenesis via regulation of PPAR-γ[19]. In the current experiment, when GW9692 was incubated with ginsenoside Rb1 stimulated adipocytes, we found that the browning specific genes (UCP-1, PGC-1α and PRDM16) were tremendously reduced. Combined with previous reports, we confirmed that ginsenoside Rb1 is an agonist of PPAR-γ.

In the present work, we demonstrated that ginsenoside Rb1 increased UCP-1 mRNA expression. We are curious to know whether ginsenoside Rb1 treatment induced UCP-1 upregulation could further trigger mitochondrial respiratory capacity. Luckily, the results revealed that ginsenoside Rb1 strikingly increased basal respiration and uncoupling effect in 3T3-L1 mature adipocytes. Further, Ginsenoside Rb1 also inhibited mitochondria apoptosis in rat cardiomyocytes [34]. The results here also offer an illustration why ginsenoside Rb1 reduce the body weight in obesity animals.

Collectively, our findings here suggest that ginsenoside Rb1 may directly activates PPARγ to induce browning in 3T3-L1 adipocytes. Of more significance, ginsenoside Rb1 stimulated browning also

![Fig. 2. Ginsenoside Rb1 promoted browning via regulation of PPARγ. The mRNA expressions of UCP-1 (A), PGC-1α (B) and PRDM16 (C) in 3T3-L1 mature adipocytes. 3T3-L1 adipocytes were treated with 10 μM of ginsenoside Rb1 in the absence or presence of GW9692 (20 μM) for 24 h 3T3-L1 adipocytes were collected and RNA was extracted from the cells. Real-time PCR were performed to detect mRNA alterations between different conditions. Data represent mean ± SEM of four independent experiments. *p < 0.05 was considered significant.](image)

![Fig. 3. The effect of ginsenoside Rb1 on PPARγ activity in COS-1 cells. COS-1 cells were transfected in batch with PPARγ as described in methods. Then transfected cells were treated with either ginsenoside Rb1 (10 μM) or control. After 18 h incubation, treated cells were developed and read in 96-well luminescence Perkin Elmer EnVision multilabel plate reader. Data represent mean ± SEM of three independent experiments. *p < 0.05 was considered significant.](image)
significantly affected mitochondrial respiration function and energy metabolism. So we believed that the browning effect of ginsenoside Rb1 would be feasible to in vivo system. This will offer a better illustration to explain the anti-obesity effect of ginsenoside Rb1 and a new source of PPARγ agonists from traditional used medicinal herbs. The in vivo browning effect of ginsenoside Rb1 still warrants further investigation.

Acknowledgement

This work was supported by grants from National Natural Science Foundation of China (NSFC81274041, NSFC81273995), International cooperation projects of MOE (2011DFA30920) and Key drug development program of MOST (2012ZX09103201-005) as well as the 111 project of MOE (B07007). The funding sources have no role in study design, data analysis, drafting and submitting the article.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.09.064.

References
