Hydroxysafflor yellow A (HSYA) inhibited the proliferation and differentiation of 3T3-L1 preadipocytes

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Abstract Hydroxysafflor yellow A (HSYA), a main component of safflor yellow, has been demonstrated to prevent steroid-induced avascular necrosis of femoral head by inhibiting primary bone marrow-derived mesenchymal stromal cells adipogenic differentiation induced by steroid. In this study, we investigate the effect of HSYA on the proliferation and adipogenesis of mouse 3T3-L1 preadipocytes. The effects of HSYA on proliferation and differentiation of 3T3-L1 cells and its possible mechanism were studied by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide spectrophotometry, Oil Red O staining, intracellular triglyceride assays, real-time quantitative RT-PCR, transient transfection and dual luciferase reporter gene methods. HSYA inhibited the proliferation of 3T3-L1 preadipocytes and cell viability greatly decreased in a dose and time dependent manner.

HSYA (1 mg/l) notably reduced the amount of intracellular lipid and triglyceride content in adipocytes by 21.3 % (2.13 ± 0.36 vs 2.71 ± 0.40 , $P < 0.01$) and 22.6 % (1.33 ± 0.07 vs 1.72 ± 0.07 , $P < 0.01$) on days 8 following the differentiation, respectively. HSYA (1 mg/l) significantly increased hormone-sensitive lipase (HSL) mRNA expression and promoter activities by 2.4- and 1.55-fold, respectively ($P < 0.01$), in differentiated 3T3-L1 adipocytes. HSYA inhibits the proliferation and adipogenesis of 3T3-L1 preadipocytes. The inhibitory action of HSYA on adipogenesis may be due to the promotion of lipolytic-specific enzyme HSL expression by increasing HSL promoter activity.

Keywords Hydroxysafflor yellow A (HSYA) · Proliferation · Differentiation · 3T3-L1 preadipocytes

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Abbreviations

HSYA Hydroxysafflor yellow A
HSL Hormone-sensitive lipase

Introduction

Obesity is now a major health problem worldwide and is closely related to the incidence of type 2 diabetes, dyslipidemia, cardiovascular disease and other diseases (Bray and Bellanger 2006). Therefore,

weight loss drug development has attracted the attention of researchers around the world. Current therapeutic agents are limited in efficacy, whereas treatment-associated adverse events have meant many agents being withdrawn. Traditional Chinese medicine is gradually being paid close attention due to its beneficial features including multi-target effects, rich resources, less side effects and safety (Mao et al. 2014; Mukwaya et al. 2014). So it is necessary to explore the weight loss drugs or ingredients from traditional Chinese medicine by using modern molecular biotechnology.

Hydroxysafflor yellow A (HSYA) is the main active ingredient of *Carthamus tinctorius L* which is one kind of the safflower plant and contains flavonols, chalcones, alkaloids and other chemical components. HSYA has been demonstrated to be effective on the inhibition of platelet aggregation, thrombosis, oxidative stress and tumor angiogenesis (Liu et al. 2008, 2012). Yu et al. (2011) recently demonstrated that HSYA prevented steroid-induced avascular necrosis of femoral head by promoting the microcirculation of caput femoris and inhibiting primary BM-MSCs adipogenic differentiation induced by steroid. It is well known that BM-MSCs are multipotent progenitor cells. However, it is unclear whether or not HSYA has some influences on the proliferation and differentiation of preadipocytes. Therefore, in our present study we observed the effects of HSYA on the proliferation and adipogenesis of mouse 3T3-L1 preadipocytes and explored its possible mechanism.

Methods

Cell culture and cell experiments

The mouse 3T3-L1 preadipocytes (passage 12) were purchased from Cell Center of Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China) and cultured according to a protocol previously implemented in our labs (Zhu et al. 2006, 2013). Briefly, 3T3-L1 cells were cultured in DMEM/F12 containing 10 % FBS (Yuan Heng Sheng Ma Biotechnology Research Institute, Beijing, China) in a CO₂ incubator. The growth state of cells was observed under an inverted phase contrast microscope.

Determination of proliferation of 3T3-L1 preadipocytes by cell counting and MTT assay

Cell counting and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay were used to evaluate cell proliferation. For cell counting assay, cells were seeded in 24-well plates with a density of 2×10^4 /ml in DMEM supplemented with 10 % FBS. After 24 h, cells were washed with phosphate-buffered saline and then exposed to various concentration of HSYA (a kind gift from Prof. Ming Jin, Department of Pharmacology, Beijing Anzhen Hospital, Capital Medical University, Beijing, China). Four, 8, 24, 48, 72, 96 h later, Trypan Blue dye and the hemacytometer were used to calculate cell number and viability. For MTT assay, 3T3-L1 preadipocytes were plated in 96-well plate and treated using similar process as cell counting. Cell growth rate was determined by MTT assay by adding MTT solution into each well with a final concentration of 1 mg/ml for 4 h. Then 100 µl triple solution (SDS 10 g, iso-butanol 5 ml and 10 N HCL 0.1 ml dissolved in double distilled water to 100 ml) was added and cells were incubated overnight. Finally, the optical density (OD) value was obtained by using an ELISA reader (Anthos Labtec, Salzburg, Austria) at a wavelength of 620 nm.

Oil Red O staining and triglycerides contents determination in 3T3-L1 cells

3T3-L1 preadipocytes were plated in 24-well plate. The differentiation was induced by treating confluent preadipocytes in 10 % FBS DMEM/F12 medium with 10 µg/ml insulin, 10 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Day 0) (Sigma, St. Louis, MO, USA). After 3 days, the medium was replaced with 10 % FBS DMEM-F12 that contained 10 µg/ml insulin for further 2 days. The medium was then changed to 10 % FBS DMEM-F12 (Day 5) and refreshed every 1–2 days. During the differentiation, these cells were treated with 0, 0.01 and 1 mg/l HSYA for 4, 8, 12 days. Oil Red O staining was conducted as described previously (Zhu et al. 2006, 2013). Briefly, the cells were fixed with 4 % fresh formaldehyde (Sigma) for 1 h at room temperature, then stained with 0.6 % (w/v) filtered Oil red O solution (Ameresco, Solon, OH, USA) for 2 h. Stained lipid droplets in cytoplasm were visualized by an inverted microscope and then photographed. Then 600 µl isopropanol was added to

extract oil red O dyes, followed by removing 150 µl extracting solution to detect the OD value by using an ELISA reader at a wavelength of 490 nm. For the determination of the contents of triglycerides in adipocytes, the cells were treated with 0, 0.01, 1 and 100 mg/l HSYA for 8 days, then lysed according to the instructions of commercial triglycerides GPO-POD enzymatic assay kit (Beijing SinoPCR, Beijing, China) and OD values were obtained by an ELISA reader. The total cell protein concentration was estimated with the BCA protein assay reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Intracellular lipid content was normalized against the protein content. Experiments were replicated at least three times.

Lipolysis assay

Cells were cultured in a 24-well plate and treated with different concentration of HSYA as described in the above triglycerides determination experiments. The medium was collected and incubated at 70 °C for 10 min to inactivate residual lipases. Glycerol released into the medium was determined by the glycerol assay kit (GPO Trinder reaction kit from Applygen Technologies Inc., Beijing, China) at 490 nm. The total protein concentration was estimated by the BCA method. Experiments were replicated at least three times. Lipolysis data were expressed as µmol of glycerol/mg of total protein.

Real-time fluorescence quantitative RT-PCR (RT-qPCR) analysis

Quantitative real-time RT-PCR (qRT-PCR) was performed with SYBR green fluorescent dye using an ABI7500 PCR system (Applied Biosystems, Foster City, CA, USA) as previously described (Gong et al. 2009). In brief, 3T3-L1 preadipocytes were plated in 24-well plate with a density of 2×10^4 /ml, then differentiated and treated with HSYA as above. Then total cellular RNA was isolated using an E.Z.N.A Total RNA Kit I (Omega Biotek, Norcross, GA, USA, Lot R6834-01). 0.5 µg of total RNA was used to produce cDNA using an RT-PCR system including Omniscript RT kit (Qiagen, Valencia, CA, USA, Lot 205111), Oligo (dT) primer (Promega, Madison, WI, USA, capsule, Lot C110A) and RNA enzyme inhibitor (Promega, capsule, Lot N251A). The primer sequences of target gene mHSL (NM_001039507) and internal control gene m18S (NR_003278) are listed in

Table 1. Total reaction volume of each well was 20 µl in 96-well plate, and each gene was repeated in duplicate. The reaction condition for PCR was initial denaturation at 95 °C for 10 min, (95 °C for 15 s, 60 °C for 1 min) $\times$ 40 cycles, and the reaction condition for dissociation curve was 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Dissociation curve of every gene demonstrated specific amplification. HSL mRNA expression level in arbitrary unit were acquired from the value of the threshold cycle (Ct) of the RT-PCR as related to that of m18S using the comparative Ct method through the formula $2^{-\Delta\Delta C_T}$ ($C_t = C_{t_{\text{gene of interest}}} - C_{t_{\text{m18S}}}$) (Livak and Schmittgen 2001). House keeping gene m18S was used as internal control to normalize the expression of target gene. The variation of the expression of 18S under various culture conditions was relative stable. Experiments were replicated at least three times.

Plasmid transfection and dual luciferase reporter assay system

On day four following cell differentiation, 0.8 µg pGL3-hHSL(−679 to +53)-luc [a firefly luciferase reporter gene expression vector which contains the human HSL (hHSL) gene 5'-promoter fragment from −679 to +53 bp and was constructed as described previously] (Zhu et al. 2013) and 0.2 µg internal control plasmid pRL-SV40 (a renilla luciferase expression plasmid purchased from Promega Corp.) were transfected into adipocytes with a proportion of plasmid DNA and transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA, Lot 11660-019) as 1 µg to 1.5 µl. After incubation of cells for 5 h, the medium was changed to 10 % FBS DMEM/F12 containing 0, 0.01 and 1 mg/l HSYA. 48 h later, the cells were lysed and both firefly and renilla luciferase activities were measured by using a commercially available Dual-Luciferase[®] Reporter Assay System kit (Promega, USA) in an automated optical immunoassay analyzer (Beijing Pilot Biotechnology Co., Beijing, China) as previously described (Gong et al. 2006). The firefly luciferase activities were adjusted using the renilla luciferase activities.

Statistical analysis

All experiment were repeated at least three times. All statistical computations were run on SPSS 13.0 for

Table 1 Primers used for real-time PCR

Genes	Primers	Sequences (5'–3')	Size (bp)
mHSL (NM_001039507)	Forward	ACTGAGATTGAGGTGCTGTC	138
	Reverse	AGGTGAGATGGTAACTGTGAG	
m18s (NR_003278)	Forward	ATGCGGCGGCGTTATTCC	194
	Reverse	CTGTCAATCCTGTCCGTGTCC

Fig. 1 Inhibitory effects of HSYA on 3T3-L1 cell viability examined by cell counting assay. The cells in 24-well plate were stimulated with 10 % FBS with different concentrations of HSYA (0, 0.01, 0.1, 1, 10 mg/l, $n = 3$) for 4, 8, 24, 48, 72, 96 h. After incubation, cell viability was determined by trypan blue staining. Fractional viability was calculated through dividing the number of clear cells by the total number of cells. Cell viability in control cells treated without HSYA was defined as 100 %. * $P < 0.05$; # $P < 0.01$ compared with the control group (0 mg/l)

Windows (SPSS Inc, Chicago, IL, USA). For cell proliferation assay, Chi square test was used to compare cell viability. For cell differentiation, mRNA expression and the promoter activity experiment, one way ANOVA was used to analyze the differences between groups. $P < 0.05$ was considered as statistically significant in all analyses.

Results

The effects of HSYA on 3T3-L1 preadipocytes proliferation

Firstly, Trypan blue assay was performed to measure the viability of 3T3-L1 preadipocytes cultured in medium containing 10 % FBS and treated with

Fig. 2 Inhibitory effects of HSYA on 3T3-L1 cell growth evaluated by MTT assay. 3T3-L1 cells were plated in 96-well plates at a density of 2×10^4 cells/ml. After 24 h, these cells were treated with HSYA (0, 0.01, 0.1, 1, 10 mg/l, $n = 9$). Cell proliferation was monitored using the MTT method at 4, 8, 24, 48, 72, 96 h following treatment. OD 620 value in 3T3-L1 cells treated without HSYA was defined as 100 %. OD optical density. * $P < 0.05$; # $P < 0.01$ compared with the control group (0 mg/l)

different doses of HSYA (0.01–10 mg/l) for different time periods (4–96 h). As shown in Fig. 1, HSYA could significantly inhibit the cell viability in a dose and time dependent manner. The maximal inhibitory action was observed in 10 mg/l HSYA treatment for 96 h where HSYA reduced cell viability by 18.9 % (80.1 ± 3.6 vs 98.7 ± 1.7 %, $P < 0.01$) in comparison with the control group. A similar phenomenon was observed for cell growth rate monitored by MTT assays which showed that HSYA gradually and significantly inhibited 3T3-L1 cell growth. With the extension of action time and an increase of HSYA concentration, the inhibitory role of HSYA was further enhanced (Fig. 2, * $P < 0.05$, # $P < 0.01$). Cell growth rate was reduced by 13.9 % in 0.1 mg/l HSYA treatment for 72 h (86.1 ± 3.7 vs 100.0 ± 1.3 %, $P < 0.01$).

Fig. 3 Intracellular lipid content determined by Oil Red O staining. 3T3-L1 cells were seeded in 24-well culture plate at a density of 2×10^4 cells/ml. The cells were differentiated and treated with 0, 0.01 and 1 mg/l HSYA for 4, 8, 12 days. Oil Red O staining were conducted as described in the “Methods” section. The total cell protein concentration was estimated by the BCA method. Intracellular lipid content was normalized against the protein. OD optical density. The data represent the mean $\pm$ SD from three independent experiments. * $P < 0.05$; # $P < 0.01$ compared with the control group (0 mg/l)

Intracellular lipid content determined by Oil Red O staining

Oil Red O could specifically stain the lipid droplets in the cytoplasm of differentiated 3T3-L1 cells. Oil Red O staining could indirectly measure the intracellular lipid content. In the present study, intracellular lipid content was normalized against cellular protein. As shown in Fig. 3, intracellular lipid content per mg of protein significantly decreased when 3T3-L1 cells were treated with different concentration of HSYA for 4, 8 and 12 days (* $P < 0.05$, # $P < 0.01$). 1 mg/l HSYA could greatly reduce the intracellular lipid content by 37.4 % (1.07 ± 0.12 vs 1.71 ± 0.29 , $P < 0.01$), 21.3 % (2.13 ± 0.36 vs 2.71 ± 0.40 , $P < 0.01$) and 27.5 % (2.68 ± 0.34 vs 3.69 ± 0.40 , $P < 0.01$) on day 4, 8 and 12 following the differentiation, respectively, in comparison with the control group.

Intracellular triglyceride content determined by triglycerides GPO-POD enzymatic assay

As shown in Fig. 4, intracellular triglyceride/mg of protein was significantly reduced when 3T3-L1 cells were treated with different concentration of HSYA

Fig. 4 Intracellular triglyceride content determined by triglycerides GPO-POD enzymatic assay. The 3T3-L1 cells were seeded and differentiated similar to the above Oil Red O staining experiments (see: Fig. 3). The cells were then treated with 0, 0.01, 1 and 100 mg/l HSYA for 8 days. The triglycerides content assay was conducted as described in the “Methods” section and triglycerides content was also normalized against the cellular protein content. OD optical density. The data represent the mean $\pm$ SD from three independent experiments. # $P < 0.01$ compared with the control group (0 mg/l)

(# $P < 0.01$). Compared with the control group, 1 and 100 mg/l HSYA could reduce the intracellular triglyceride content by 22.6 % (1.33 ± 0.07 vs 1.72 ± 0.07 , $P < 0.01$) and 28.0 % (1.24 ± 0.09 vs 1.72 ± 0.07 , $P < 0.01$) on day eight following differentiation, respectively.

Glycerol released into the medium determined by the glycerol assay kit

The level of glycerol released in the medium of 3T3-L1 adipocytes is generally used to assess the lipolytic effect. In this study, 3T3-L1 preadipocytes were differentiated and treated with different concentrations of HSYA. As shown in Fig. 5, glycerol/mg of total protein in the medium of 3T3-L1 cells treated with 1 and 100 mg/l HSYA for 8 days was significantly increased by 40.7 and 104.1 % in comparison with the control group (0.51 ± 0.04 vs 0.36 ± 0.07 ; 0.74 ± 0.05 vs 0.36 ± 0.07 , $P < 0.01$, respectively).

HSYA elevates HSL mRNA level in 3T3-L1 adipocytes

As depicted in Fig. 6, the expression level of HSL mRNA was significantly increased when 3T3-L1 cells

Fig. 5 Glycerol released into the medium determined by the glycerol assay kit. Cells were cultured in a 24-well plate and treated with different concentrations of HSYA as described in the triglycerides determination experiments (see: Fig. 4). Glycerol released into the medium was determined by the glycerol assay kit (GPO Trinder reaction) at 490 nm. The total protein concentration was estimated by the BCA method. Lipolysis data were expressed as micromoles of glycerol released per milligram of total cellular protein. Experiments were replicated at least three times. [#] $P < 0.01$ compared with the control group (0 mg/l)

Fig. 6 HSYA elevated HSL mRNA level in 3T3-L1 adipocytes. 3T3-L1 cells were seeded in 24-well plate at a density of 2×10^4 cells/ml. The cells were differentiated and treated with 0, 0.01, 1 and 100 mg/l HSYA for 8 days. The total RNA was extracted and real-time RT-PCR was conducted as described in the “Methods” section. All results were normalized to the values that were obtained for the 18S control, and the results are expressed as fold changes of the Ct value relative to the control value, which was defined as 1. The data represent the mean $\pm$ SD from three independent experiments. ^{*} $P < 0.05$; [#] $P < 0.01$ compared with the control group (0 mg/l)

were treated with different concentrations of HSYA for 8 days. 0.01, 1 and 100 mg/l HSYA could notably increase the expression level of HSL mRNA to 2.2 ($P < 0.05$), 2.4 ($P < 0.01$) and 2.7 ($P < 0.01$) fold on

Fig. 7 HSYA enhanced HSL promoter activity in 3T3-L1 adipocytes. The 3T3-L1 cells were differentiated using a differentiation cocktail medium. On day four following differentiation, a luciferase reporter gene plasmid pGL3-hHSL(−679 to +53)-luc (0.8 μg/well, $n = 3$) and an internal control plasmid pRL-SV40 (0.2 μg/well) were transfected into adipocytes using Lipofectamine 2000, and the cells were treated with 0.01 and 1 mg/l HSYA. 48 h later, the cells were lysed, and both the firefly and renilla luciferase activities were measured. The firefly luciferase activities were adjusted using the renilla luciferase activities. The data represent the mean $\pm$ SEM from three independent experiments. [#] $P < 0.01$ compared with the control group (0 mg/l)

day 8 following the differentiation, respectively, in comparison with the control group.

HSYA enhances the HSL promoter activity in 3T3-L1 adipocytes

pGL3-hHSL(−679 to +53)-luc and pRL-SV40 plasmids were transiently transfected into 3T3-L1 adipocytes on the 4th day of differentiation which were treated with HSYA at the same time. Two days later, the HSL promoter activity was detected by dual luciferase[®] Reporter Assay System. The results showed that the relative means of HSL promoter activity in cells treated with 0.01 and 1 mg/l HSYA were elevated to 128 and 155 %, respectively, in comparison with the control group (Fig. 7, $P < 0.01$).

Discussion

Safflower yellow has been widely used in clinical practice in the treatment of cardiovascular and cerebrovascular ischemic disease including coronary heart disease and cerebral thrombosis. HSYA, as the main

active ingredient of the safflower plant (*C. tinctorius* L), has two solution structures of keto-enol tautomers (2a and 2b). Keto-enol tautomers 2a is the preferred tautomer with the 1-enol-3,7-diketo form (Feng et al. 2013). It has been demonstrated that HSYA can antagonize platelet aggregation and inhibit thrombosis. In the present study, we found that HSYA inhibited the proliferation of 3T3-L1 preadipocytes and cell viability greatly decreased in a dose and time dependent manner, suggesting HSYA inhibited 3T3-L1 proliferation by inducing cell death. Consistent with our results, Ji et al. (2008) and Xi et al. (2009) demonstrated that HSYA inhibited the proliferation of human umbilical vein endothelial cells and angiogenesis by reducing basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and VEGF-receptor (VEGF-R) mRNA expression in vitro. Wang et al. (2013) demonstrated that HSYA inhibited the proliferation and extracellular matrix production of liver astroid cells by stimulating PPAR γ activity. Conversely, Zhang et al. (2008) found that HSYA promoted the proliferation of canine thoracic aortic endothelial cells by regulating VEGF and VEGF-R expression. In addition, Tu et al. (2010) demonstrated that higher concentrations (>0.0657 g/L) of HSYA inhibited the proliferation of human aortic endothelial cells, while lower concentrations (<0.0657 g/L) of HSYA promoted cell proliferation. These inconsistent results may be due to the various cell types or the different HSYA concentrations with different effects.

Our study also showed that treatment of adipocytes with HSYA significantly reduced the observed level of Oil-Red O staining and TG content, indicating a decrease in the number of lipid droplets in cytoplasm and a reduction in the size of adipocytes. A similar result was obtained by Yu et al. (2011) who demonstrated the triglycerides levels in steroid-induced BM-MSCs were significantly lower than in the control group after treatment with HSYA. All these findings suggested that HSYA could inhibit adipogenesis of adipocytes. In order to further investigate the mechanism by which HSYA reduced lipid accumulation in 3T3-L1 cells, glycerol content released into the medium of 3T3-L1 adipocytes treated with HSYA were analyzed and it was found that HSYA could significantly promote lipolysis in adipocytes. Further study showed that HSYA promoted lipolysis

through increasing HSL mRNA expression by activating HSL promoter activity. Studies performed by Large et al. (1999) showed that the lipolysis capacity and HSL mRNA and protein levels in subcutaneous adipose tissue were all significantly reduced in obese patients in comparison with non-obese subjects. Furthermore, expressions of HSL mRNA and protein in cachexia patients increased by 50 % and 1–2.5-fold, respectively, compared with both weight-stable cancer controls and nonmalignant controls (Agustsson et al. 2007; Cao et al. 2010). All of these findings indicated that HSYA could suppress the content of triglycerides in adipocytes by promoting the lipolysis, and will have beneficial action in the anti-obesity field.

HSYA is the major component of flavonoids in safflower and has a structure of chalcone. Hsu and Yen (2007) demonstrated that the antioxidants including flavonoids and phenolic acids were able to significantly inhibit the generation of triglycerides in adipocytes and the activity of glycerol-3-phosphate dehydrogenase which is the key enzyme in lipogenesis. Similarly, Liu et al. (2007) reported that the flavonoid naringin could inhibit adipogenesis of 3T3-L1 preadipocytes by reducing PPAR γ and C/EBP α expression. Hsieh et al. (2012) also found that chalcone derivatives had potential anti-diabetic activities. Xanthohumol, the chalcone from beer hops, greatly inhibit differentiation of preadipocytes (Yang et al. 2007). These results, together with the recent finding that HSYA might provide protection against heart and cerebral ischemia injury through its antioxidant action (Liu et al. 2008; Wei et al. 2005), suggested that HSYA could overcome certain metabolic alterations that are associated with the obese state and be a promising target for anti-obesity and anti-cardiovascular ischemic disease therapies.

In conclusion, HSYA inhibited the proliferation and adipogenesis of 3T3-L1 preadipocytes. The inhibitory action of HSYA on adipogenesis may be due to the promotion of the expression of the lipolytic-specific enzyme HSL by increasing HSL promoter activity.

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Conflict of interest There is no conflict of interest.

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