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Danshen extract 15,16-dihydrotanshinone I functions as a potential modulator against metabolic syndrome through multi-target pathways

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ABSTRACT

Hypertension is a common complication of type 2 diabetes mellitus (T2DM), and is the main cause for T2DM-associated mortality. Although the stringent control of blood pressure is known to be beneficial in reducing the cardiovascular mortality of T2DM patients, drugs with both anti-hypertensive and anti-hyperglycemic effects are seldom reported. The traditional Chinese medicine *danshen* has long been used for lowering both blood pressure and blood glucose in T2DM patients, shedding lights on the development of such medication. However, the molecular mechanism and active component remain unclear. Here, we report that the lipophilic component, 15,16-dihydrotanshinone I (DHTH) from *danshen* potently antagonized both mineralocorticoid and glucocorticoid receptors, and efficiently inhibited the expression of their target genes like Na^+/K^+ ATPase, glucose 6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK). In addition, DHTH increased AMPKα phosphorylation and regulated its downstream pathways, including increasing acetyl-CoA carboxylase (ACC) phosphorylation, inhibiting transducer of regulated CREB activity 2 (TORC2) translocation and promoting glucose uptake. Such discovered multi-target effects of DHTH are expected to have provided additional understandings on the molecular basis of the therapeutic effects of *danshen* against the metabolic syndrome.

1. Introduction

Cardiovascular disease is an extremely common morbidity and the main cause for the mortality of type 2 diabetes mellitus (T2DM). Notably up to 75% of cardiovascular disease in diabetes may be attributable to hypertension [1]. The mechanisms for T2DM-associated hypertension are complicated. It is believed that the enhanced sodium re-absorption in the renal tubules and the resultant increased sodium retention in the body caused by hyperinsulinaemia mainly account for the T2DM-induced hypertension [2]. Numerous clinical data suggest that rigorous control of blood pressure (BP) could reduce the incidence of cardiovascular complications and effectively decrease fatality of T2DM patients [3–5]. Therefore, captopril, atenolol, ramipril or other BP lowering drugs are frequently employed in the treatment of T2DM in combination with anti-hyperglycemic reagents or insulin sensitizers. However, due to the risk of drug–drug interactions in the combination therapy, drugs with both BP lowering and anti-hyperglycemic effects integrated into a single chemical entity are needed.

Danshen, the dried root of *Salvia miltiorrhiza*, is a traditional Chinese medicine that has been used for hundreds of years, with the conventional application in the treatment of cardiovascular disease including hypertension [6]. Notably, this herb was recently found to efficiently improve insulin sensitivity, lower blood glucose level and alleviate diabetic nephropathy in T2DM patients [7,8]. In order to clarify the potential molecular therapeutic mechanism for *danshen*, its components and biological activities were extensively studied. Generally, the chemical constituents of *danshen* include lipophilic and hydrophilic components, of which cryptotanshinone, tanshinone I and tanshinone IIA are the major lipophilic components, while salvianic acid A, protocatechuic aldehyde and salvianolic acid B are the major hydrophilic components [9]. Recently, lithospermic acid B, the hydrophilic component of *danshen*, was found to inhibit angiotensin converting enzyme (ACE), and markedly attenuated angiotensin I-induced vasoconstriction [10–12], which might partially explain the anti-hypertension effect of *danshen*. However, the contribution of the lipophilic components to *danshen*'s therapeutic effect was poorly understood.

In the current work, we discovered that 15,16-dihydrotanshinone I (DHTH, Fig. 1a), a lipophilic component of *danshen*, functioned as antagonists of both mineralocorticoid and glucocorticoid receptors, and inhibited the expression of their target genes, such as glucose 6-phosphatase (*G6Pase*), phosphoenolpyruvate carboxykinase (*PEPCK*) and *Na^+/K^+ ATPase*.

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Besides, DHTH was also identified as an AMPK pathway activator, which could increase AMPKα phosphorylation and activate its downstream pathways, including increasing acetyl-CoA carboxylase (ACC) phosphorylation, inhibiting transducer of regulated CREB activity 2 (TORC2) translocation and promoting glucose uptake. Our findings have provided one possible understanding for the anti-hypertension and anti-T2DM effects of *danshen*, while DHTH could serve as a promising lead compound for further development in the treatment of T2DM associated with hypertension.

2. Materials and methods

2.1. Reagents

15,16-Dihydrotanshinone I was isolated from *Salvia miltiorrhiza* as described in supplementary method. Cytochalasin B, 2-deoxy-D-glucose, biotin, 3-isobutyl-1-methylxanthine, dexamethasone, ionomycin, *p*-nitrophenyl-galactopyranoside and forskolin were purchased from Sigma–Aldrich. Pantothenic acid calcium salt was purchased from Eastman. 2-[^{3}H]-Deoxy-D-glucose was purchased from PerkinElmer. Cell culture reagents were all bought from GIBCO. Hoechst 33342 was obtained from Invitrogen. All antibodies were purchased from Cell Signaling Technology except anti-GAPDH antibody (KangChen, China). Dual Luciferase Assay System was purchased from Promega. ATP Analysis Kit was obtained from Beyotime (Shanghai, China). TRIzol reagent was from Generay Biotech (Shanghai, China). PrimeScriptTM RT reagent Kit was obtained from TaKaRa (Japan). SYBR Green Real-time PCR master mix was from TOYOBO (Japan).

2.2. Plasmids

pGL3-GRE/MRE-Luc was constructed by inserting 2× GRE/MRE sequence (TGTACAGGATGTTCTctctgcctctgcTGTACAGGATGTTCT) into pGL3-promoter vector. pSuper.neo.gfp-LKB1 plasmid was constructed by inserting the LKB1-siRNA sequence (cgaagagaagcagaaaatg) [13] into BglII-HidIII sites.

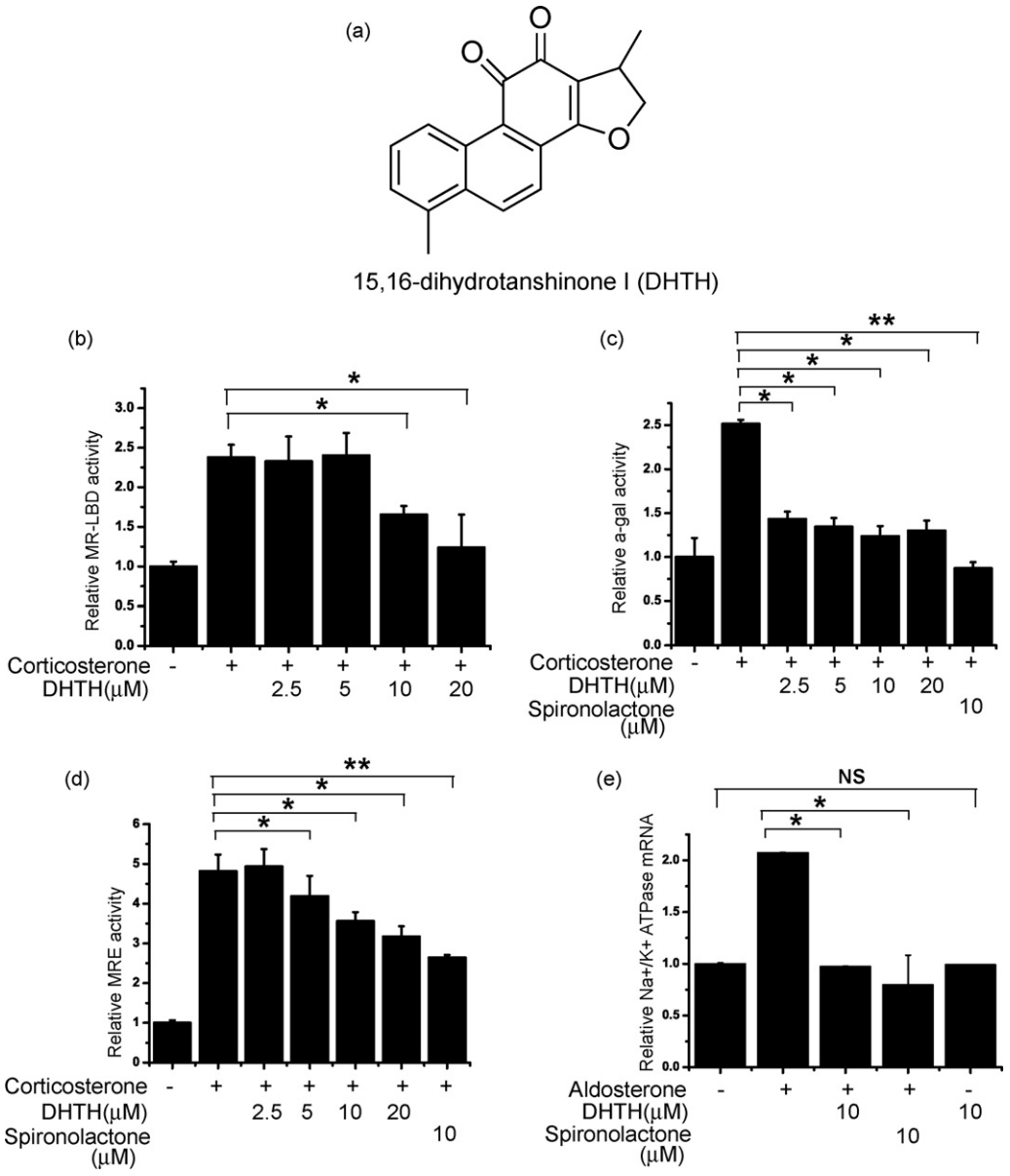

Fig. 1. DHTH is an MR antagonist. (a) Chemical structure of 15,16-dihydrotanshinone I (DHTH). (b) DHTH inhibited MR-LBD activation. (c) DHTH blocked MR-SRC1 interaction. (d) DHTH inhibited MRE transactivation. (e) DHTH inhibited *Na$^+$/K$^+$ ATPase* expression. Significant difference at $^*P < 0.05$, $^{**}P < 0.01$, NS: no significant difference, $n = 3$.

2.3. Cell culture and differentiation

U2-OS/TORC2-GFP, U2-OS/GR-GFP (Bioimage, Denmark), U2-OS, HEK293T, HK-2, and MIN6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/ml). U2-OS-siLKB1 cell line was generated by transient transfection of U2-OS cells with pSuper.neo.gfp-LKB1 plasmid or empty vector, and screened with G418 for 2 weeks. Clones with green fluorescence were selected with clone rings. The stable cell lines si-1 and si-2 carry two different LKB1-siRNA sequences. 3T3-L1 adipocytes were cultured in DMEM supplemented with 10% FBS, penicillin–streptomycin, biotin (8 mg/l) and pantothenic acid calcium salt (4 mg/l). All the cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The differentiation procedure of 3T3-L1 cells followed the classic method. Briefly, 2 days after 100% confluence, cells were stimulated with MDI cocktail (115 mg/l methylisobutylxanthine, 0.39 mg/l dexamethasone and 1 mg/l insulin) for 3 days, and medium was then replaced with fresh medium containing 1 mg/l insulin for another 3 days.

2.4. Western blotting

Cells were incubated with DHTH (5, 10, 20, 30 μM, 4 h), AICAR (1 mM, 4 h), or ionomycin (2 μM, 5 min) respectively and harvested using SDS-PAGE sample buffer (60 mM Tris–HCl, 2% SDS, 4 mM EDTA, 5% β-mercaptoethanol, 0.02% bromophenol blue and 10% glycerol, pH 8.0). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Proteins were detected with corresponding antibodies and signal developed with west-dura substrate (Pierce). Experiments were repeated for three times, and bands were quantified by Image-Pro Plus software. Phosphorylated AMPK levels were normalized by total AMPK and GAPDH or β-tubulin. Phosphorylated ACC levels were normalized by GAPDH or β-tubulin. Data was expressed as fold of control.

2.5. Nuclear translocation assay

Nuclear translocation assay of GR was performed using U2-OS/GR-GFP cell line. Cells were incubated with DHTH for 4 h in phenol red-free DMEM supplemented with 5% charcoal stripped FBS, stimulated with dexamethasone (5 nM, 0.5 h) and meanwhile nucleus was stained with hoechst 33342 (2 μM, 0.5 h). Fluorescence pictures were obtained using INCell Analyzer 1000 (GE Healthcare) and data was analyzed with the nuclear translocation module. For determination of IC_{50} values, each treatment was repeated in 4 wells, and pictures of 8 fields were taken for each well. The ratio of fluorescence intensity in the nuclear and cytoplasm was calculated by the INCell Analyzer analysis software, and expressed as Nuc/Cyto GR to indicate nuclear location of GR. The experiment was repeated for three times.

Nuclear translocation assay of TORC2 was performed using the U2-OS/TORC2-GFP stable cell line. Cells were incubated with indicated concentrations of DHTH, stimulated with forskolin (0.8 μM, 0.5 h), fixed and nucleus were stained with hoechst 33342 (2 μM, 0.5 h). The IC_{50} value was calculated as that described for GR translocation.

2.6. Mammalian one-hybrid assay and transactivation assay

Mammalian one-hybrid assay was used to investigate the effects of DHTH on GR-LBD and MR-LBD. HEK-293T cells were co-transfected with pUAS-TK-Luc reporter plasmid, pRL-SV40 and the fusion constructs of Gal4DBD-GR-LBD, or Gal4DBD-MR-LBD, respectively. Cells were then treated with indicated concentrations of DHTH (0, 2.5, 5, 10, 20 μM) and DEX (20 nM) or corticosterone (20 nM) for 24 h, and luciferase activity was measured with Dual Luciferase Assay kit. The result was presented as fold activation related to untreated cells after normalization with Renilla luciferase values, which was unaffected in the assays. Each experiment was repeated for three times, with each sample analyzed in triplicates.

Mammalian transactivation assay was performed to detect the antagonistic effect of DHTH on MR/GR transcriptional activity. HEK293T cells transfected with plasmids for GR transactivation (pGL3-GRE-Luc, pRL-SV40 and pCI-nGFP-GR(C656G)) or MR (pGL3-MRE-Luc, pRL-SV40 and pGFP-rMR) were incubated with indicated concentrations of DHTH (0, 2.5, 5, 10, 20 μM) and DEX (20 nM) or corticosterone (20 nM) for 24 h. Luciferase activities were measured as described above.

2.7. Measurement of promoter activity

For evaluation of the antagonistic effects of DHTH on *PEPCK* and *G6Pase* promoters, HepG2 cells were transfected with pGL3-*PEPCK*-luc or pGL3-*G6Pase*-luc with pRL-SV40, and then incubated with DHTH (20 μM), GR antagonist RU486 (1 μM) and DEX (20 nM) for 24 h. Luciferase activity was measured using Dual Luciferase Assay kit.

2.8. Yeas two-hybrid assay

AH109 yeast cells were co-transfected with pGBKT7-MR-$LBD_{725–981}$ and pGADT7-SRC-$1_{613–773}$ plasmids. Stable transformants (OD_{600} = 0.05) were incubated with indicated concentrations of DHTH (0, 2.5, 5, 10, 20 μM) or spironolactone (10 μM) in the presence of corticosterone (20 nM) for 24 h, and α-galactosidase activity was then measured using *p*-nitrophenyl-galactopyranoside as the substrate.

2.9. Real-time PCR

HK-2 cells were incubated with 20 nM aldosterone with or without 10 μM DHTH or 10 μM spironolactone for 24 h. Total RNA was extracted with TRIzol reagent. Complementary cDNA was synthesized using PrimeScriptTM RT reagent Kit. Real-time PCR was performed using SYBR Green Real-time PCR master mix using DNA Engine Opticon 2 System (Bio-Rad Laboratories, USA). The PCR cycle was 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. The primer pairs fare as follows. *Na^+/K^+ ATPase*-α1: (F): 5′-GACATGACCTCCGAGCAGC-3′ (R): 5′-GACGACCTTCCTCTACTCC-AG-3′; 18sRNA: (F): 5′-GTAACCCGTTGAACCCCATT-3′ (R): 5′-CC-ATCCAATCGGTAGTAGCG-3′; LKB1: (F): 5′-GAGCTGATGTCGGT-GGGTATG-3′ (R): 5′-CACCTTGCCGTAAGAGCCT-3′.

2.10. Cellular ATP level measurement

U2-OS cells were treated with 20 μM DHTH for 4 h, and intracellular ATP was measured via the luciferin/luciferase method using an ATP Analysis Kit (Beyotime, China). Briefly, cells were washed with ice-cold PBS and lysed. Luminescence was determined by a GENios microplate reader (Tecan, Switzerland). Cellular protein concentrations were determined using a BCA Protein Assay Kit (Pierce, USA). ATP contents were calculated according to a standard curve of the supplied ATP standards and normalized using the cellular proteins.

2.11. 2-Deoxyglucose uptake

3T3-L1 cells were seeded into 24-well plates and fully differentiated (the 10th day after MDI stimulation). The glucose uptake assay was performed according to the previous report [14] with modifications. Briefly, after incubation of the compound (DHTH, 5, 10, 20 μM or AICAR, 1 mM) in the serum-free medium containing

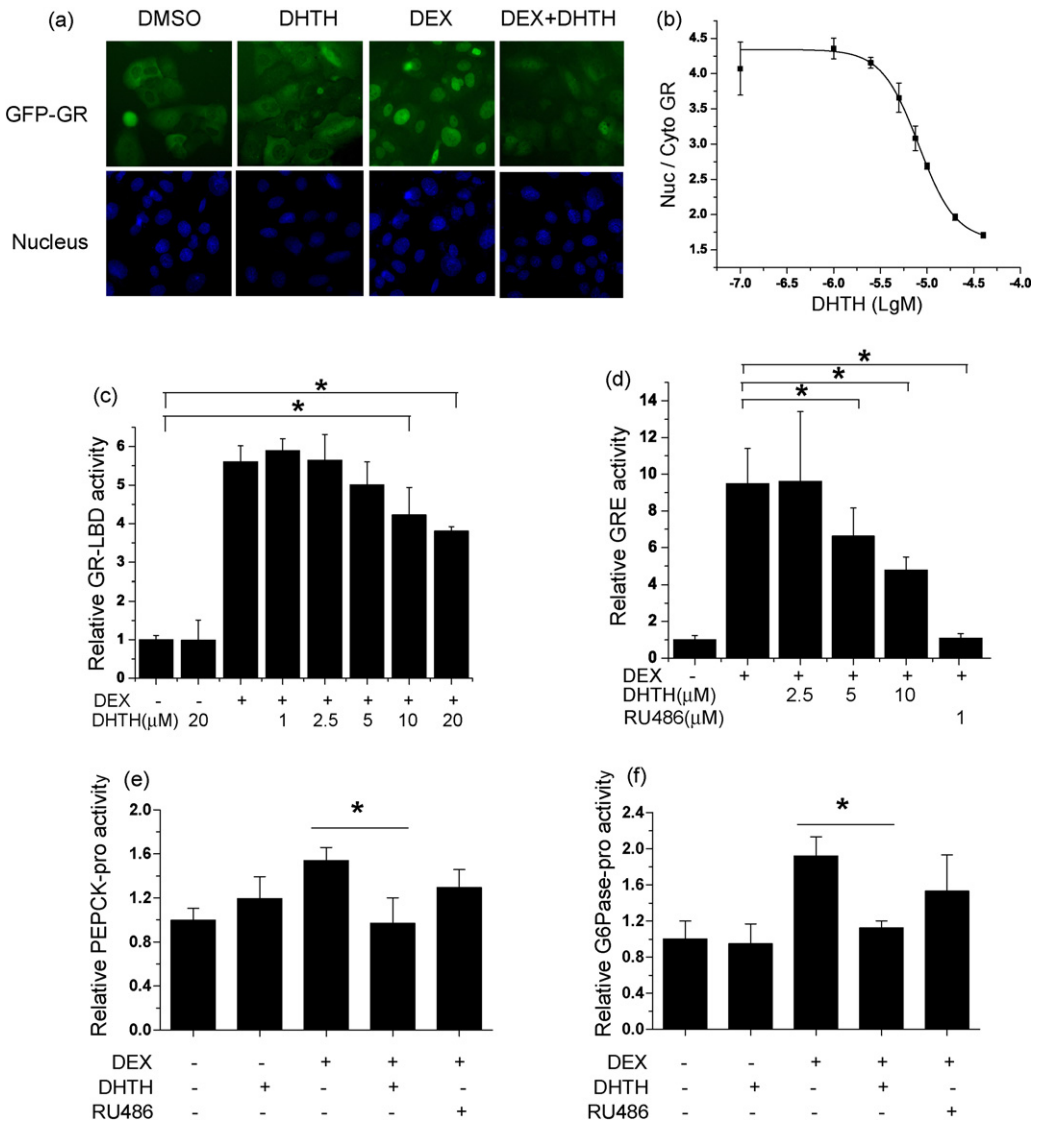

Fig. 2. DHTH is a GR antagonist. (a) DHTH inhibited GR nuclear translocation with (b) IC_{50} of 8.1 μM. (c) DHTH inhibited GR-LBD activation. (d) DHTH inhibited GRE transactivation. (e and f) DHTH inhibited (e) *PEPCK* and (f) *G6Pase* mRNA expression. Significant difference at $^*P < 0.05$, $n = 3$. Scale bar, 10 μm.

0.5% BSA for 4 h, the medium was replaced with Krebs buffer and cells were incubated for another 0.5 h. In the last 5 min, cells were incubated with 2-[^{3}H]-deoxy-D-glucose. The glucose uptake was stopped by ice-cold PBS and cells were lysed with 0.1% Triton X-100. The radioactivity was finally calculated by a scintillation counter. Cytochalasin B was used to measure nonspecific uptake and the value was subtracted from all data.

3. Results

3.1. DHTH is a mineralocorticoid receptor (MR) antagonist

MR antagonists (*e.g.* eplerenone and spironolactone) have been widely used as anti-hypertension drugs [15]. The potential antagonistic effect of DHTH on MR was investigated using the mammalian one-hybrid assay in HEK293T cells (human embryonic kidney cell line), which were frequently used in reporter gene assays for its high transfection efficiency. The cells were transiently transfected with expression plasmids for GAL4-DBD-MR-LBD and luciferase reporter gene driven by UAS-response element for 24 h, followed by incubation with corticosterone (20 nM) in the presence or absence of DHTH (2.5, 5, 10, 20 μM) for 24 h. As shown in Fig. 1b, DHTH dose-dependently antagonized the activation of MR-LBD induced by the endogenous MR agonist corticosterone.

As reported, the activated nuclear receptors might recruit co-activators and subsequently initiate the transcription program [16]. We thereby tested whether DHTH could block the co-activator recruitment of MR using yeast two-hybrid assay, which may reduce unspecific interactions compared with mammalian cells. The plasmids pGADT7-SRC-1 and pGBKT7-MR-LBD were stably transfected into yeast strain AH109, and the stable transformants were incubated with corticosterone (20 nM) in the presence or absence of DHTH (2.5, 5, 10 and 20 μM) for 24 h, with spironolactone (10 μM) as a positive control. The results (Fig. 1c) indicated that DHTH could potently inhibit the corticosterone-induced interaction between MR-LBD and SRC-1.

Mammalian transactivation assay was performed to further verify the antagonistic effect of DHTH on MR. HEK293T cells were transiently transfected with an MR expression plasmid and a pGL3-MRE-Luc reporter plasmid for 24 h, followed by incubation with corticosterone (20 nM) alone or corticosterone and DHTH (2.5, 5, 10, 20 μM) for another 24 h. As expected, DHTH exhibited dose-dependent inhibition against corticosterone-induced transcriptional activation of MR (Fig. 1d).

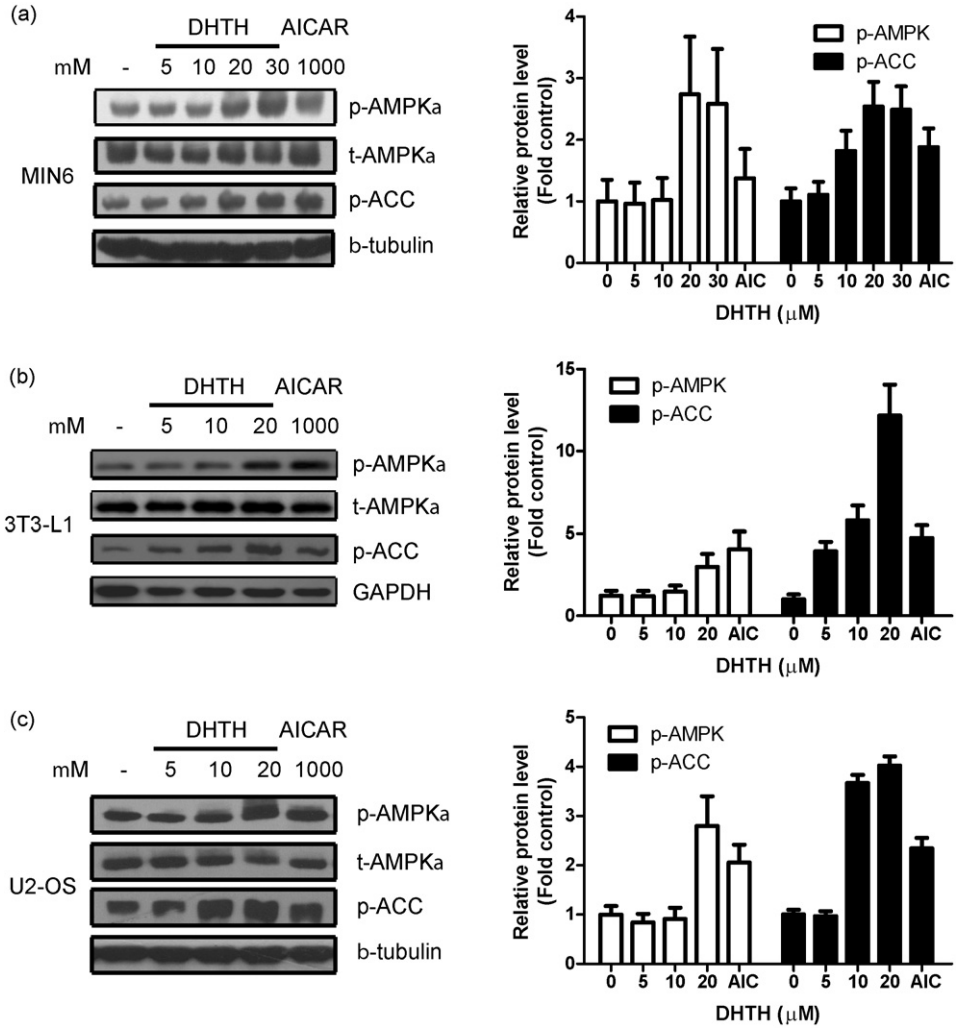

Fig. 3. DHTH increased AMPKα phosphorylation in (a) MIN6, (b) 3T3-L1 and (c) U2-OS cells. AMPKα and ACC phosphorylation levels and total AMPKα level were determined by Western blotting. GAPDH or β-tubulin were used as loading controls. The results shown are representative of three independent experiments (left panel). The bands were quantified using Image-Pro Plus software. Protein levels were normalized by total AMPK, GPADH or β-tubulin and expressed as fold of control (right panel).

3.2. DHTH strongly inhibited the aldosterone-induced expression of Na^+/K^+-ATPase gene

Na^+/K^+-ATPase is one of the MR regulated genes and its abnormal expression in kidney epithelial cells is highly involved in the pathogenesis of hypertension [17,18]. Since DHTH was discovered as an MR antagonist, the inhibitory effect of DHTH on *Na^+/K^+-ATPase* expression was analyzed by quantitative real-time PCR. The proximal tubular epithelial cell line HK-2, which is derived from normal kidney was used in this experiment. As indicated in Fig. 1e, consistent with previous reports, aldosterone induced about 2-fold increase of *Na^+/K^+-ATPase* expression [19], DHTH (10 μM) could completely block the induction. Spironolactone (10 μM) was taken as a positive control in the assay.

3.3. DHTH is a glucocorticoid receptor (GR) antagonist

As reported, GR mainly distributed in the cytoplasm and translocated to the nucleus upon ligand binding [20]. Therefore, a U2-OS/GR-GFP stable cell line was used to study the effect of DHTH on the intracellular distribution of GR. Following the previous reports [21], GR was retained in the cytoplasm without stimulation, while DEX significantly induced its nuclear translocation (Fig. 2a). DHTH exhibited no effects on the cellular distribution of GR by itself, but dose-dependently inhibited the DEX-induced GR nuclear translocation (Fig. 2a) with an IC_{50} value of 8.1 μM (Fig. 2b).

To further evaluate the antagonistic activity of DHTH against glucocorticoid receptor (GR), mammalian one-hybrid assay was performed. HEK293T cells transfected with Gal4-DBD-GR-LBD expression plasmid and luciferase reporter plasmid were incubated with DHTH (1, 2.5, 5, 10, 20 μM) in the presence of the GR agonist dexamethasone (DEX, 20 nM) [22] for 24 h. As indicated in Fig. 2c, DHTH significantly antagonized the DEX-induced GR-LBD activation.

Considering that DHTH could inhibit both nuclear translocation and LBD activation of GR, it is expected that DHTH might antagonize GR transcriptional activity. To prove this point, mammalian transactivation assay was performed in HEK293T cells. Cells transfected with GR expression plasmid and GRE (GR response element) luciferase reporter plasmid were incubated with indicated concentrations of DHTH and DEX for 24 h. As shown in Fig. 2d, DHTH dose-dependently antagonized the DEX-stimulated GR transcriptional activity. The GR antagonist RU486 was used as a positive control [23].

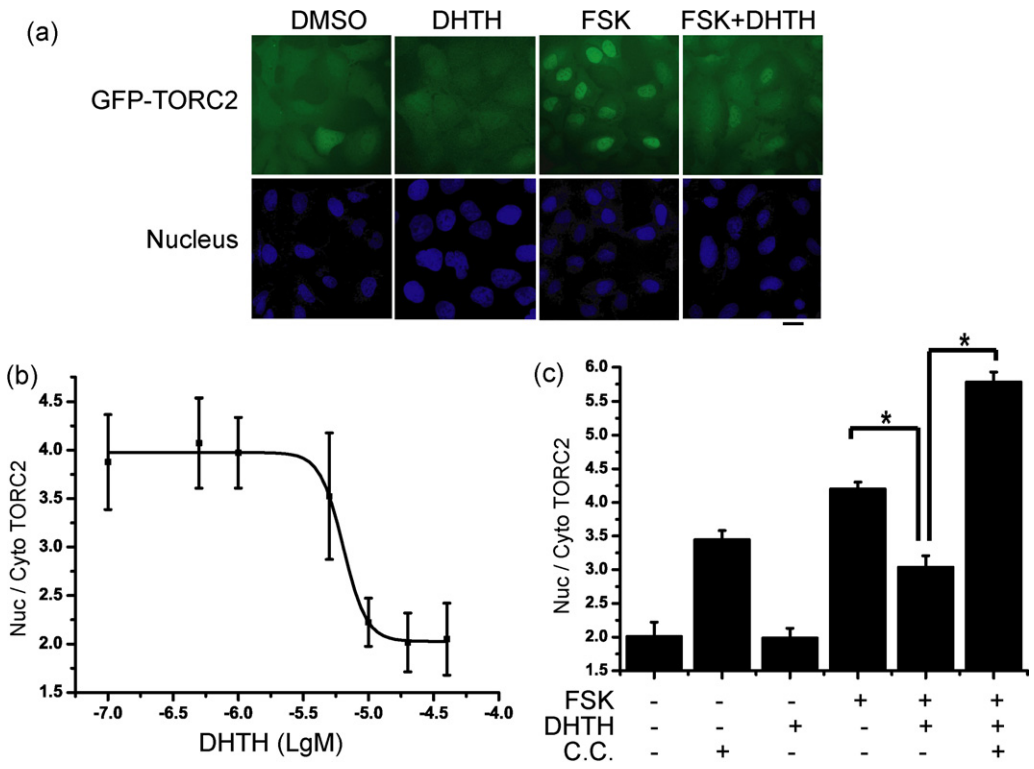

Fig. 4. DHTH inhibited TORC2 translocation. (a) DHTH inhibited TORC2 nuclear translocation in with (b) IC_{50} of 6.4 μM. (c) Compound C (C.C.) abolished the inhibition of DHTH on TORC2 nuclear translocation. Significant difference at $^*P < 0.05$, $n = 6$. Scale bar, 10 μm.

3.4. DHTH significantly inhibited the expression of PEPCK and G6Pase genes

By considering that the activated GR could increase the expression of PEPCK and G6Pase [24], which are key enzymes involved in hepatic gluconeogenesis, the potential inhibitory effects of DHTH on *PEPCK* and *G6Pase* expression were thus investigated. HepG2 hepatoblastoma cells derived from the liver tissue were used for study of hepatic gluconeogenesis. The cells were transfected with luciferase reporter plasmids containing *PEPCK* or *G6Pase* promoter or 24 h, and were subsequently incubated with indicated concentrations of DHTH and DEX for 24 h. As shown in Fig. 2e and f, DEX increased *PEPCK* and *G6Pase* promoter activity by 60 and 100%, respectively, while DHTH could almost completely block these increases.

3.5. DHTH efficiently activated the AMPK pathway

3.5.1. DHTH dose-dependently increased AMPKα phosphorylation

The AMP-activated protein kinase (AMPK) is the cellular energy sensor and has been implicated as a promising drug target against metabolic syndrome [25]. Encouraged by the recent finding that another lipophilic component of *danshen*, cryptotanshinone functioned as an anti-hyperglycemic agent by activating AMPK pathway [26], we thereby explored the possibility whether DHTH could also activate this pathway. As expected, DHTH dose-dependently activated AMPKα phosphorylation in MIN6 insulinoma cells, 3T3-L1 adipocytes and U2-OS osteosarcoma cells (Fig. 3). The reported AMPK activator, AICAR was used as a positive control.

3.5.2. DHTH increased ACC phosphorylation

As reported, AMPK controls lipid and glucose metabolism in multiple peripheral tissues [25]. Activation of AMPK could expedite body lipid oxidation, thus inhibit lipogenesis. This effect was mainly mediated by acetyl-CoA carboxylase (ACC), which was inactivated after phosphorylation by AMPK at Ser 79. Therefore, we examined the effects of DHTH on ACC phosphorylation. As shown in Fig. 3, DHTH dose-dependently increased ACC phosphorylation in MIN6, 3T3-L1 and U2-OS cells.

3.5.3. DHTH inhibited TORC2 translocation through activation of AMPK

Since AMPK could also suppress hepatic gluconeogenesis by inhibiting nuclear translocation of TORC2 [27,28], the potential effects of DHTH on TORC2 translocation were also explored using the U2-OS/TORC2-EGFP stable cell line. As reported, TORC2 distributed both in the cytoplasm and nucleus in resting cells, and forskolin significantly increased its nuclear translocation [28] (Fig. 4a). DHTH had no effects on the distribution of TORC2 by itself, but dose-dependently inhibited forskolin-induced nuclear translocation of TORC2 with an IC_{50} value of 6.4 μM (Fig. 4a and b). Furthermore, the AMPK inhibitor, compound C (C.C., 10 μM) could completely reverse the inhibition of DHTH on TORC2 translocation (Fig. 4c). These results thus indicated that DHTH inhibited TORC2 translocation through activation of AMPK.

3.5.4. DHTH dose-dependently promoted glucose uptake in 3T3-L1 cells

AMPK could increase glucose uptake both in adipose and skeletal muscle tissues [29]. Thereby, we studied the possible effects of DHTH on glucose uptake in fully differentiated 3T3-L1 adipocytes. As expected, DHTH dose-dependently promoted glucose uptake in 3T3-L1 adipocytes (Fig. 5).

3.6. DHTH enhanced AMPKα phosphorylation through a CaMKK and LKB1-independent pathway

To further explore the possible signaling pathways responsible for DHTH-induced AMPK activation, effects of DHTH on the

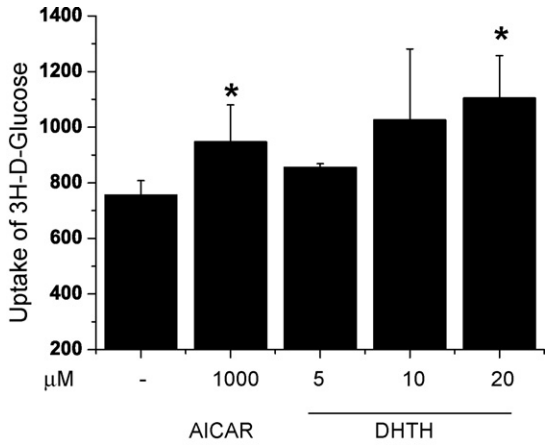

Fig. 5. DHTH increased glucose uptake. Fully differentiated 3T3-L1 cells were stimulated with 1 mM AICAR or indicated concentrations of DHTH for 4 h. In the last 5 min, cells were incubated with 2-[^{3}H]-deoxy-D-glucose. The radioactivity was calculated by a scintillation counter. Significant difference at $^*P < 0.05$, $n = 4$.

two reported AMPKα kinase, LKB1 and calmodulin-dependent protein kinase kinase (CaMKK) [25] are studied. The activation of AMPKα by LKB1 was regulated by cellular AMP/ATP ratio. Our study showed that the intracellular ATP level did not change even after 4-h incubation with DHTH (Fig. 6a), which was adequate to activate AMPKα. What's more, stable knock-down of LKB1 did not affect DHTH-induced AMPKα phosphorylation (Fig. 6b and c). In addition, stimulation of AMPK phosphorylation by AICAR was decreased in the two stable cell lines si-1 and si-2 (Fig. S2). These results thereby suggested that LKB1 was not involved in DHTH-induced AMPKα phosphorylation.

The activity of CaMKK was elevated by the increase of intracellular calcium. To study the possible role of CaMKK in DHTH-induced AMPKα phosphorylation, the CaMKK inhibitor STO609 was incubated with DHTH. The results showed that STO609 did not affect DHTH-induced AMPKα phosphorylation, although it completely blocked ionomycin (a calcium ionophore) induced AMPKα phosphorylation (Fig. 6d and e). The above results indicated that DHTH may possibly stimulate AMPKα phosphorylation via a CaMKK and LKB1-independent pathway.

4. Discussion

Hypertension is one of the most prevalent complications of T2DM [1], and many T2DM patients have to rely on additional drugs for tight control of blood pressure. However, the current treatments against such syndrome are all based on the combination therapies, in which two or three BP lowering drugs are combined with anti-T2DM drugs. It is believed that the single drug with multi-target mechanism could simplify the clinical trial and reduce the risk of drug–drug interactions compared with the combination therapy. The anti-hypertension and anti-T2DM therapeutic effects of *danshen* have encouraged us to study the molecular mechanism and identify the possible active components.

In the present work, the multi-target mechanism of DHTH, a lipophilic component of *danshen* was studied. DHTH was discovered to be an MR antagonist and inhibited *Na^+/K^+ ATPase* expression, which may contribute to the anti-hypertension effects of *danshen*. Meanwhile, DHTH also showed antagonistic effect on GR activation, and inhibited *PEPCK* and *G6Pase* expression, the key enzymes in gluconeogenesis. In addition, DHTH increased AMPKα phosphorylation and regulated its downstream signaling pathways. DHTH inhibited TORC2 translocation, which was an important transcriptional regulator for *PEPCK* and *G6Pase*. In addition to inhibition of gluconeogenesis, DHTH also promoted glucose uptake which may together help to improve hyperglycemic state. Finally, DHTH increased ACC phosphorylation which may lead to decreased lipogenesis (Fig. 7).

Aldosterone, a physiological ligand of MR, mainly targets epithelia in the kidney and colon, and plays a fundamental role in electrolyte and fluid homeostasis, thereby controlling the blood pressure. It activates MR and subsequently induces the expression of a set of genes including sodium channel (ENaC) [30], glucocorticoid-inducible kinase (Sgk) [31], corticosteroid hormone-induced factor (CHIF) [32], Na^+/K^+ ATPase [19], *etc.* MR over-activation results in not only hypertension, but also cardiovascular and renal injury. Therefore, inhibition of MR has become a

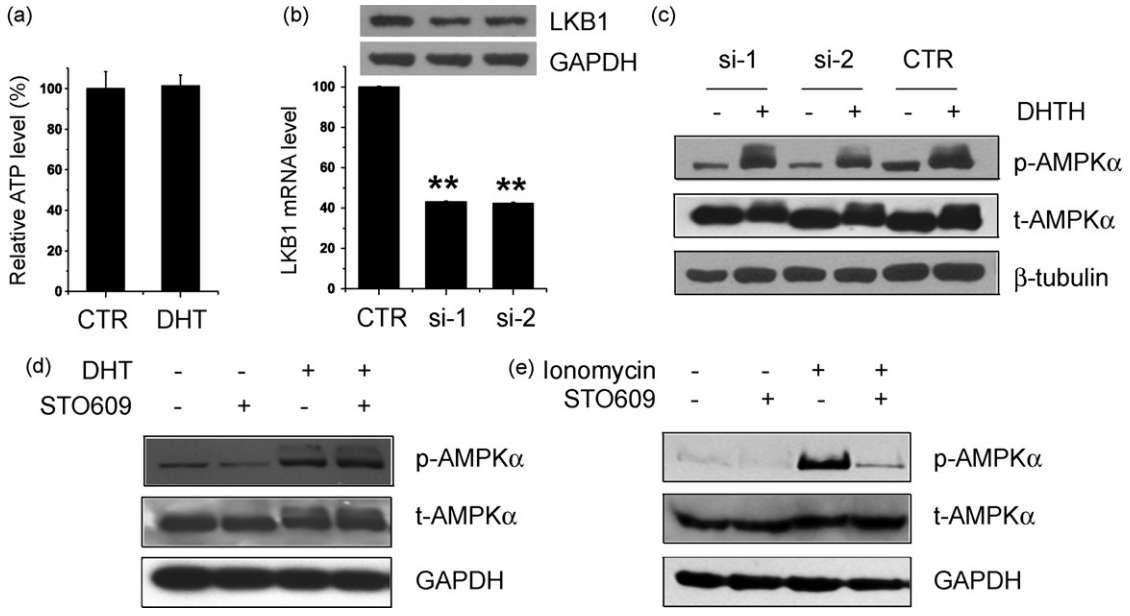

Fig. 6. DHTH increased AMPKα phosphorylation by a LKB1 and CaMKK-independent pathway. (a) DHTH did not affect cellular ATP level. (b) LKB1 mRNA levels were determined in the LKB1 knock-down stable cell lines. (c) DHTH increased AMPKα phosphorylation in LKB1 knock-down cells. (d) The CaMKK inhibitor STO609 did not affect DHTH-induced AMPKα phosphorylation. (e) STO609 completely blocked ionomycin-induced AMPKα phosphorylation.

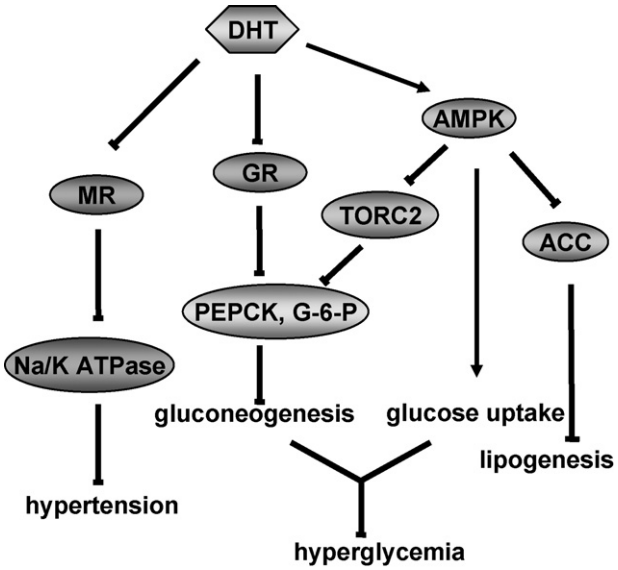

Fig. 7. Proposed model illustrating the anti-T2DM molecular mechanism of DHTH. DHTH antagonized MR activity and inhibited *Na^+/K^+ ATPase* expression, which results in anti-hypertension effects. Meanwhile, DHTH antagonized GR activation, and inhibited *PEPCK* and *G6Pase* expression, the key enzymes in gluconeogenesis. In addition, DHTH increased AMPKα phosphorylation and regulated its downstream signaling pathways, which finally lead to anti-hyperglycemic effects and reduced lipogenesis.

promising strategy to treat these syndromes, and the two MR antagonists, spironolactone and eplerenone have already been clinically used for several years [33]. In our study, the fact that DHTH could dose-dependently inhibit activation of MR-LBD, block the interaction between MR-LBD and SRC1, and inhibit MR transcriptional activity has proved DHTH to be a potent MR antagonist. By antagonizing MR-LBD, DHTH significantly inhibited aldosterone-induced expression of *Na^+/K^+ ATPase*. Considering that MR antagonization is beneficial to BP control, the intrinsic MR-antagonism characteristic of DHTH is expected to partially account for the anti-hypertension activity of the herb *danshen*.

Chronic excessive activation of GR induces obesity, insulin resistance, hypertension, *etc*. The most representative disease is the Cushing's syndrome, which is caused by a high level of blood glucocorticoid and results in central obesity, insulin resistance, hyperglycemia, dyslipidaemia and hypertension [34]. Based on the phenotypic similarities between patients with Cushing's syndrome and patients with metabolic syndrome or T2DM, it is expected that inhibiting glucocorticoid action might be a promising strategy for the treatment of metabolic syndrome and T2DM [35]. In our work, DHTH was found to be a potent GR antagonist, as evidenced by its inhibition of DEX-induced GR-LBD activation, GR nuclear translocation, as well as *PEPCK* and *G6Pase* expression. The GR antagonistic activity of DHTH might partially account for the anti-T2DM effect of *danshen*. In addition, to investigate the selectivity of DHTH among steroid hormone receptors, the effect of DHTH on LXR, FXR, PR, and RXR was also investigated using mammalian one-hybrid assays. Our results indicated that DHTH showed no agonistic or antagonistic activity on these receptors (data not shown).

In our work, the mammalian one-hybrid assay indicated that DHTH blocked the agonist induced GR and MR LBD activation, while the yeast two-hybrid assay results revealed that DHTH blocked the agonist induced SRC1 recruitment by MR. These results thus implied that DHTH was likely to directly interact with GR and MR LBD. The chemical structure of DHTH resembles that of the steroidal ligands of mineralocorticoid and glucocorticoid, however, whether the antagonists compete with agonist binding was rarely investigated, since antagonistic activity of nuclear receptor ligands was generally exerted by altered co-factor recruitment [36]. Concerning the molecular interaction, Pandit et al. reported that the GR antagonist RU486 slowed the dissociation rate of GR from DNA [37].

AMPK is a cellular energy sensor and has been considered as a promising drug target for the treatment of T2DM [25]. This protein controls cellular glucose and lipid metabolism in several peripheral tissues including liver, adipose tissue, skeletal muscle and pancreas. For example, AMPK could stimulate glucose uptake in skeletal muscle and adipose tissue [38], suppress gluconeogenesis by inhibiting TORC2 nuclear translocation in liver, and inhibit lipogenesis by phosphorylating ACC in liver and skeletal muscle. Recently, AMPK is found to mediate the anti-diabetic and anti-obesity effects of metformin and rosiglitazone. Therefore, identification of new compounds that could activate AMPK pathway would contribute to the treatment of T2DM. The discovered DHTH could dose-dependently increase AMPKα phosphorylation, and its effects on AMPK downstream pathways were also investigated. We found that DHTH increased ACC phosphorylation in MIN6, U2-OS and 3T3-L1 cells, inhibited TORC2 nuclear translocation in U2-OS/TORC2 cells, and promoted glucose uptake in 3T3-L1 cells. Such a finding that DHTH could significantly activate AMPK pathway might provide additional pharmacological understanding for the therapeutic effects of *danshen* on T2DM.

In our work, the potential mechanism for DHTH-induced AMPK activation was further investigated. To date, AMPKα phosphorylation is reported to be mainly regulated by two protein kinases, LKB1 and CaMKK [25]. In the state of low cellular ATP level, AMP binding induces AMPK conformation change and subsequent phosphorylation by LKB1, while phosphorylation of AMPKα by CaMKK is promoted by an increase of intracellular calcium level. Accordingly, we investigated whether these two kinases were possibly involved in DHTH-induced AMPK activation. Surprisingly, DHTH had no effect on cellular ATP level, and could increase AMPKα phosphorylation in LKB1 stable knock-down U2-OS cells, thus indicating that DHTH-induced AMPKα phosphorylation was not mediated by LKB1. Moreover, the result that the CaMKK inhibitor STO609 did not affect DHTH-induced AMPKα phosphorylation also excluded the possible involvement of CaMKK. Therefore, these results implied that DHTH increases AMPKα phosphorylation via a CaMKK and LKB1-independent pathway, which deserves further investigation.

In conclusion, we have explored the potential multi-target mechanism of a lipophilic component, 15,16-dihydrotanshinone I (DHTH), from *danshen*. DHTH functioned as a potent antagonist of MR/GR and an effective activator of the AMPK pathway. Our results may contribute to the understandings of molecular mechanisms for the anti-hypertension and anti-T2DM activity of *danshen*, while DHTH might assist the development of therapeutic reagents for the treatment of T2DM associated with hypertension.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.03.090.

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