A Natural Compound Formononetin Derived From *Astragalus Membranaceus* Increase Adipocyte Thermogenesis by Modulating PPARγ Activity

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.14139

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Running title: Formononetin Promotes Adipocyte Thermogenesis

ABSTRACT

BACKGROUND AND PURPOSE

Increasing energy expenditure through adipocyte thermogenesis is generally accepted as a promising strategy to mitigate obesity and its related diseases, however, few effective and safe agents are available clinically that promote adipocyte thermogenesis. In this study, 20 traditional Chinese herbal medicines were screened to examine whether they induced adipocyte thermogenesis.

EXPERIMENTAL APPROACH

The effects of Chinese herbal medicines or indicated components from *Astragalus membranaceus* on adipocyte thermogenesis were analyzed by qPCR. 8 week old C57BL6/J male mice were fed on high fat diets for 8 weeks and then randomized in two groups treated with vehicle or formononetin for 8 weeks. Glucose tolerance test and adipose tissue H&E staining were performed. Whole-body oxygen consumption was measured with an open-circuit indirect calorimetry system.

KEY RESULTS

Astragalus membranaceus was found to promote adipocyte thermogenesis *in vitro*. Formononetin in *Astragalus membranaceus* was then identified as the critical constituent for increasing adipocyte thermogenesis. Furthermore, mice treated with formononetin also showed less bodyweight gain and higher energy expenditure than the control mice. In addition, formononetin was found to bind directly

with PPARγ in the biochemical analysis. These results suggest that formononetin regulates adipocyte thermogenesis as a partial PPARγ agonist.

CONCLUSIONS AND IMPLICATION

Taken together, our study demonstrates that the Chinese herbal medicine *Astragalus membranaceus* and its constituent formononetin have the potential to reduce obesity and associated metabolic disorders.

Tables of Links

TARGETS[a,b]	
Nuclear hormone receptors[a]	RXR
PPARα	RAR
PPARγ	**Transporters**[b]
β3-adrenoceptor	UCP1

LIGANDS[c]	
Rosiglitazone	Bexarotene
CL316243	AM580
WY14643	

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 ([a,b,c] Alexander *et al.*, 2015a,b,c).

Abbreviations:

PPARγ, peroxisome proliferator-activated receptor-γ; UCP1, uncoupling protein 1; BAT, brown adipose tissue; WAT, white adipose tissue.

Introduction

Obesity is becoming increasingly prevalent worldwide, leading to numerous metabolic diseases (Di Cesare *et al.*, 2016). As energy imbalance is the main cause of obesity, it is effective to treat obesity by reducing energy intake or increasing energy expenditure. Uncoupling protein 1 (UCP1) can convert energy into heat to increase energy expenditure for non-shivering thermogenesis. As UCP1 is mainly expressed in the brown and brite/beige adipocytes, increasing UCP1 expression or activity in adipocytes is anticipated as a promising strategy to prevent and treat obesity (Cypess *et al.*, 2013; Feldmann *et al.*, 2009).

Brite/beige adipocytes can be induced in the white adipose tissue and their contribution to thermogenesis was estimated to equal to that of brown adipocytes (Shabalina *et al.*, 2013). The developmental origins of brown and beige adipocytes are different, but the core mechanism that regulates UCP1 expression is believed to be the same (Bartelt *et al.*, 2014). Peroxisome proliferator-activated receptor-γ (PPARγ) is a key transcriptional factor for regulating the expression of *Ucp1* by directly binding to its promoter (Villarroya *et al.*, 2007). Chronic activation of PPARγ by its full agonist rosiglitazone can significantly induce UCP1 expression in primary epididymal adipocytes (Petrovic *et al.*, 2010). Rosiglitazone but not partial agonists of PPARγ can stabilize the critical co-activator protein PR domain containing 16 (PRDM16) to promote expression of *Ucp1* (Ohno *et al.*, 2012). PRDM16 not only determines the identity of brown fat cells but also controls the formation of beige adipocytes (Cohen *et al.*, 2014; Seale *et al.*, 2008).

Many cytokines or drugs have been demonstrated to treat obesity by increasing adipocyte thermogenesis, but a critical problem is that few people like to regard obesity as an illness and treat it medicinally (Bi *et al.*, 2014; Buemann *et al.*, 2000; Fisher *et al.*, 2012; Wang *et al.*, 2015; Zhang *et al.*,

2014; Baskaran *et al.*, 2016). Many traditional Chinese herbal medicines have been widely used to maintain daily health for hundreds of years. Therefore, 20 traditional Chinese herbal medicines were screened to examine whether they had the potential to induce adipocyte thermogenesis for the treatment of obesity. In this study, *Astragalus membranaceus* and its constituent formononetin were found to be involved in adipocyte thermogenesis via the regulation of PPARγ activity.

Methods

Isolation of adipocytes and stromal vascular cells from adipose tissues

Adipose tissues were dissected from C57BL/6J mice, rinsed in phosphate-buffered saline, minced, and digested for 40 min at 37^{o}C in a 0.1% (w/v) type I collagenase solution (Sigma) with a D-Hanks buffer. The digested tissue was filtered through a 250-μm nylon mesh and centrifuged at 800 x g for 3 min. The sediment was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (HyClone). Two days after reaching confluence (day 0), the cells were induced to differentiate into adipocytes in a medium containing 5 μg/ml insulin (Sigma), 1 μM of dexamethasone (Sigma), 0.5 mM of isobutylmethylxanthine (Calbiochem), and 1 μM of rosiglitazone (Sigma). Two days later, the medium was replaced with DMEM supplemented with 10% fetal bovine serum, 5 μg/ml of insulin, and 1 μM of rosiglitazone, and the cells were cultured for 6 days.

Chinese herbal medicine extract

1kg of *Astragalus membranaceus* was added to 10L of water and boiled for 2 hours, and then the first water extract was harvested. The sediment was next added to 8,000 ml of water and boiled for 1.5 hours, after which the second water extract was harvested. The first and second water extracts were mixed and concentrated to 500 ml by boiling. The concentration was 2.0 g/ml of *Astragalus*

membranaceus water extract. The following Chinese herbal medicines were obtained by the same method: *Cichorium intybus* (1.5 g/ml), *Cinnamomum cassia* (0.3 g/ml), *Coix lachryma-jobi* (1.0 g/ml), *Zingiber officinale* (1 g/ml), *Eucommia ulmoides* (1.2 g/ml), *Eugennia caryophyllata* (0.2 g/ml), *Sophora japonica* (0.8 g/ml), *Hippophae rhamnoides* (0.42 g/ml), *Alpinia officinarum* (0.5 g/ml), *Lonicera japonica* (1 g/ml), *Morus alba* (1.2 g/ml), *Astragalus membranaceus* (2 g/ml), *Atractylodes lancea* (0.6 g/ml), *Lycium barbarum* (1.2 g/ml), *Piper nigrum* (0.1 g/ml), *Sterculia lychnophora* (1 g/ml), *Morus alba* (0.8 g/ml), *Citrus reticulata* (0.6 g/ml), *Euodia rutaecarpa* (0.3 g/ml), and *Trigonella foenum-graecum* (0.8 g/ml). The doses of all the indicated Chinese herbal medicine were referred in Pharmacopoeia of the People's Republic of China 2015. Water extracts of *Astragalus membranaceus* were dissolved in methanol, and the mixture was then analyzed on a reverse-phase high performance liquid chromatography (HPLC) and elution times of the mixture were compared with pure formononetin (Figure S2). HPLC instrument is Dionex Summit HPLC on a 4.6 × 250 mm Inertsil column with ODS-SP, 5.0 μm from GL Sciences Inc. HPLC was run with a mobile phase: CH_3CN and H_2O (32: 68), a flow rate of 1.0 mL/min and detection wavelength of 254 nm. Differentiated mature adipocytes were treated in DMEM plus 10% FBS with the addition of the vehicle or an indicated Chinese herbal medicine water extract or indicated agents (*Astragalus membranaceus* polysaccharide [MCE], astragaloside IV [Sigma], formononetin [Selleck], calycosin [MCE], StemRegenin 1 [SR1, Selleck]) for 24 h. After that, the cells were harvested for real-time qPCR analysis.

RNA extraction and quantitative PCR

Total RNA was isolated with Trizol (Invitrogen), and first-strand cDNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen) with 0.5 μg of RNA as the template for each

reaction. The mRNA levels were quantified under optimized conditions with SYBR Premix Ex Taq (Takara Bio) following the manufacturer's instructions. The reference gene was 18S ribosomal RNA as an internal standard. The average mRNA levels of the genes were normalized to the control adipocytes or the adipose tissues in the control mice.

Mice

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health (GIBH), Chinese Academy of Sciences. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2015). C57BL/6J mice under high fat diet were routinely used as obesity mouse models (Bi *et al.*, 2014; Dominguez *et al.*, 2014). Eight-week-old male C57BL/6J mice were maintained on a 12-h light/12-h dark cycle at 23° C in a specific pathogen free environment. Mice were allowed access to different diets and water *ad libitum* and housed in groups of four in separate cages. The mice were fed on a high fat diet (D12492, Research Diets) for 8 weeks to become obese, and then randomly divided into two groups and gavaged for the following 8 weeks with either *Astragalus membranaceus* water extract (3.3g/kg) or formononetin (50mg/kg) and vehicle (control) on a high fat diet. The dose of *Astragalus membranaceus* in mice was referred in Pharmacopoeia of the People's Republic of China 2015. The diet provided 21.9 kJ/g: 60% of energy from fat, 20% from protein, and 20% from carbohydrate. Food intake and body weights were measured weekly. The rectal temperature was measured at 12 h after cold exposure (4° C). After feeding the mice were sacrificed by cervical dislocation. These results were analyzed by an investigator blinded to the treatments of the mice.

Histology

Brown, inguinal, and epididymal adipose tissues were fixed in 4% formaldehyde overnight at room temperature, embedded in paraffin, and cut into 5-μm sections with a microtome. The slides were deparaffinized, rehydrated, and stained with hematoxylin and eosin (Sigma) following a standard protocol. Sections were examined by light microscopy (Motic BA600) and photographed with a Moticam Pro 285A. Photomicrographs were scanned with an Abaton Scan 300 color scanner.

Indirect calorimetry and calculated energy expenditure

Whole-body oxygen consumption was measured with an open-circuit indirect calorimetry system with automatic temperature and light controls (Comprehensive Lab Animal Monitoring System, Columbus Instruments). Mice had ad libitum access to chow and water in respiration chambers, and data were recorded for 48 h, including 24 h of acclimatization. Energy expenditure was calculated as recommended by the manufacturer.

PPARγ luciferase activity assay

The PPARγ luciferase activity assay was performed as described previously (Malapaka *et al.*, 2012). Briefly, COS-7 cells from ATCC were grown to 70% confluence in DMEM supplemented with 10% FBS and antibiotics. Then the COS-7 cells were transiently co-transfected with 100 ng of a plasmid containing the luciferase gene under the control of three tandem PPAR-response elements (PPAR-response element x 3 TK-luciferase) and 50 ng of full-length human PPARγ plasmids using Lipofectamine 2000 (Invitrogen). After 24 h, the transfected cells were treated with DMSO, formononetin, or rosiglitazone for additional 24 h. Reporter luciferase assay kits from Promega were used to measure the luciferase activity following the manufacturer's instructions with a luminometer (Turner Biosystems, CA, USA).

AlphaScreen binding assays

The human PPARγ LBD (residues 206–477) containing a His6 tag was expressed as described previously (Li *et al.*, 2008). The binding of the PGC-1α-1 peptideto PPARγ was determined by AlphaScreen assays using a hexahistidine detection kit from PerkinElmer Life Sciences. The experiments were conducted with 20 nM of His tag receptor LBD and 20 nM of biotinylated PGC1α-1 peptide in the presence of 5 μg/ml of donor and acceptor beads in a buffer containing 50 nM of MOPS, pH 7.4, 50 mM of NaF, 50 mM of CHAPS, and 0.1 mg/ml of bovine serum albumin. The biotinylated peptides of PGC-1α-1 were AEEPSLLKKLLLAPA.

SiRNA transfection

SiRNA oligonucleotides were designed and synthesized by RiboBio Company (Guangzhou, China). On day 8 of differentiation, siRNA (20 nmol) was transfected into the primary adipocytes with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Eight hours after transfection, the medium was replaced with a normal culture medium. The adipocytes were treated with the indicated agents for 48 h, or longer as indicated, and then harvested for analysis.

Seahorse analysis

The oxygen consumption rate (OCR) of the cells was analyzed using the XFe24 Seahorse bioanalyzer. One day prior to the analysis, the cells were treated with formononetin (1μM) or DMSO. After 24 h, the cells were equilibrated in sodium carbondioxide-free DMEM for 1 h in CO_2 free incubator. After measuring the basal levels of OCR, the following drugs were sequentially loaded to each well: oligomycin (5μM), FCCP (5μM), rotenone (3μM) + antimycin (5μM).

Culture of 3T3-L1 cells and induction of differentiation

The 3T3-L1 pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% (v/v) fetal bovine serum (FBS, Hyclone). Two days after the cells reached confluence (day

0), they were transferred to an MDI differentiation medium containing 5 μg/mL of insulin (Sigma), 1 μM of Dex (Sigma), 0.5 mM of IBMX (Calbiochem), and either the vehicle, 1μM of formononetin, or 1 μM of rosiglitazone. Two days later, the medium was replaced with DMEM supplemented with 10% FBS, 5 μg/mL of insulin plus, and either the vehicle, 1 μM of formononetin, or 1 μM of rosiglitazone. The cells were subsequently re-fed every two days until day 6.

For oil red O staining, the 3T3-L1 adipocytes were fixed with 10% formalin for 5 min, and then incubated in fresh formalin for 1 hour. After washing with 60% (v/v) isopropanol, the cells were stained for 10 min in freshly diluted oil red O. For quantitative analysis, oil red O was eluted with 100% isopropanol for 10 min, after which the optical density was determined at 500 nm with a spectrophotometer (Beckman Coulter).

Data and Statistical Analysis

The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015). Data are expressed as means ± SEM. ANOVA and unpaired, two-tailed t tests were used for most comparisons in GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Post hoc tests were run only when F achieved $P < 0.05$ and there was no significant variance in homogeneity. Tukey's HDS test was used for body weight gain and GTT data. To control for unwanted sources of variation, data were normalized to an internal standard (see Results for more details). $P < 0.05$ was considered significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to

PHARMACOLOGY 2017/18 (Alexander *et al.*, 2015).

Results

Formononetin derived from Astragalus membranaceus induced adipocyte thermogenesis in vitro

Because brown and beige adipocytes use the same molecular mechanism in the regulation of *Ucp1* expression, primary inguinal adipocytes were used to screen for effective Chinese herbal medicines. Isolated adipose stromal cells from the inguinal adipose tissue were differentiated into mature adipocytes *in vitro*, after which they were treated with 20 different Chinese herbal water extracts for 24 hours. The cells were then harvested for qPCR analysis. As shown in Figure 1A, *Astragalus membranaceus* extract (Astra) significantly enhanced the expression of *Ucp1* in these adipocytes. The *Astragalus membranaceus* extract comprised different constituents, including *Astragalus membranaceus* polysaccharide, astragaloside, formononetin (formo), calycosin, and so on (Agyemang *et al.*, 2013). To identify the constituents involved in adipocyte thermgeonesis, each of these constituents was used to treat the adipocytes, respectively. *Astragalus membranaceus* polysaccharide was the largest constituent in the *Astragalus membranaceus* extract, but had no effect on adipocyte thermogenesis (Figure 1B). Astragaloside IV, which has been reported to regulate adiponectin secretion in adipocytes, had no effect on adipocyte thermogenesis either (Figure 1C) (Xu *et al.*, 2009). However, formononetin, an O-methylated isoflavone, was found to increase the expression of *Ucp1* dose-dependently (Figure 1D). On the other hand, its analog calycosin had no effect on adipocyte thermogenesis (Figure 1E and S1). Furthermore, the combination of *Astragalus membranaceus* extract and formononetin had no obvious synergistic effect on expression of *Ucp1*. Additionally, formononetin elevated the expression of other thermogenic genes (Figure 1F-G). HPLC analysis

demonstrated that there was comparable amount of formononetin in water extract of *Astragalus membranaceus* (Figure S2). Thus formononetin appears to be the critical constituent that mediates the effect of *Astragalus membranaceus* extract on adipocyte thermogenesis. Immunofluorescence staining further showed that formononetin can increase the UCP1 protein level (Figure 1H). The effect of formononetin on adipocyte thermogenesis was further verified by Seahorse bioanalyzer, which indicated that treatment of formononetin is capable of increasing both basal and uncoupled mitochondrial respiration (Figure 1I).

Resistance to diet-induced obesity in mice treated with formononetin

Given that other constituents in the *Astragalus membranaceus* extract may also influence body weight in obese mice, diet-induced obese (DIO) mice were treated with only formononetin for 8 weeks. Bodyweight and food intake were measured weekly. After 4 weeks of treatment, the formononetin-treated mice showed lower bodyweight than the control mice (Figure 2A). And food intake did not differ between these two group mice (Figure 2B). MRI analysis results suggested that formononetin reduced only fat mass but not lean mass in DIO mice (Figure 2C). Consistent with this finding, the inguinal and epididymal adipose tissue weights were lower in treated mice than in the controls (Figure 2D). H&E staining analysis also confirmed that the size of the adipocytes in the adipose tissue was smaller in formononetin-treated mice than in the control mice (Figure 2E-F). Consistently, insulin sensitivity was improved in the formononetin treated mice (Figure 2G).

As expected, expression of the thermogenic genes was increased in the inguinal, epididymal, and brown adipose tissues of formononetin treated mice (Figure 2H-J). UCP1 protein expression in both the brown and inguinal adipose tissue of the formononetin treated mice was enhanced in the western blot analysis (Figure 2K and S3). Indirect calorimetry analysis proved that O_2 consumption, body

temperature, CO_2 production and energy expenditure were higher in mice treated with formononetin while no difference in locomotor activity was observed (Figure 2L-N and S4). To further delineate the molecular mechanism how formononetin helped mice to resist obesity via promoting adipocyte thermogenesis, DIO mice were placed in thermoneutral environment (30° C) where mice no longer need to activate adipocyte thermogenesis to maintain body temperature and the mice were gavaged with vehicle or formononetin for 8 weeks. We did not observe any significant difference in body weight changes at thermoneutrality between two groups (Figure S5), suggesting increased UCP1 protein alone, without sympathetic activation or cold stress, does not work to increase energy expenditure. Taken together, our results showed that formononetin, a constituent of *Astragalus membranaceus* extract, can reduce bodyweight gain in obese mice by increasing adipocyte thermogenesis.

PPARγ mediates the function of formononetin

Several studies have reported that formononetin is a potential ligand of estrogen receptors, aryl hydrocarbon receptors, and PPARα/γ (Medjakovic *et al.*, 2008). Estrogen receptors (ERs) could not be targeted by formononetin for adipocyte thermogenesis, as estrogen has been reported to have no effect on *Ucp1* induction in brown adipocytes, and the ER antagonist tamoxifen can induce thermogenesis *in vivo* (Andersen *et al.*, 2014; Hesselbarth *et al.*, 2015). In addition, the aryl hydrocarbon receptor (AhR) antagonist itself did not affect adipocyte thermogenesis and had no effect on adipocyte thermogenesis induced by formononetin (Figure S6). To test the addictive effect of formononetin with the other known thermogenesis inducers, differentiated adipocytes were treated with 1μM formononetin with

1μM AM580 (RAR agonist), 1μM Bexarotene (Bex, RXR agonist), 1μM CL316243 (β3-adrenoceptor agonist), 1μg/ml FGF21 and 1μM WY14643 (PPARα agonist) respectively and these indicated compounds alone for 24 h. In combination with other known thermogenesis inducers, formononetin had a significant synergistic effect on *Ucp1* expression with all except for rosiglitazone (Figure 3A-B). *Astragalus membranaceus* extract had no additive effect with rosiglitazone on adipocyte thermogenesis either (Figure 3C). These results suggest that formononetin have the same molecular target as rosiglitazone. Thus the PPARγ plasmid and PPRE-luciferase reporter were used to carry out PPARγ activation assay, and as expected, both formononetin and rosiglitazone dose-dependently promoted luciferase activity (Figure 3D and S7). Furthermore, siRNA knockdown of *Pparγ* dramatically reduced adipocyte thermogenesis induction by formononetin (Figure 3E and S8). To further verify that formononetin can bind directly with PPARγ, AlphaScreen binding assay was performed (Li *et al.*, 2008). In this assay, the co-activator peptide of PGC-1α and the PPARγ LBD protein were attached to donor and acceptor beads respectively. Upon interaction between the co-activator peptide of PGC-1α and the PPARγ LBD, excitation with a laser beam at 680 nm causes the donor beads to emit single oxygen molecules that activate fluorophores in the acceptor beads, and the light is recorded at 520–620 nm. The ligand for the PPARγ LBD induced the stronger interaction, and the results clearly demonstrated that formononetin can bind directly with the ligand-binding domain of PPARγ like rosiglitazone (Figure 3F). However, its PPARγ activity was only about 10% that of rosiglitazone. Rosiglitazone was a classical TZD drug for the treatment of diabetes, but it induced obesity in mice, whereas our results showed that formononetin treatment resists obesity. Obesity is accompanied by adipogenesis, and as *Pparγ* is the master gene for adipogenesis, we compared the roles of the two compounds in adipogenesis. As shown in Figure 3G-H, formononetin

promoted only mild adipogenesis compared to rosiglitazone under the MDI cocktail, and unlike rosiglitazone, it could not induce adipocyte formation in the presence of insulin alone. Thus the limited ability of formononetin on adipogenesis is likely to account for the reduced bodyweight gain compared to rosiglitazone. Based on these results, we summarized our findings in a diagram to illustrate the thermogenesis mechanism by formononetin and *Astragalus membranaceus* (Figure 4).

Discussion

TZD drugs as ligands for PPARγ were widely used to treat diabetes and promote insulin sensitivity, but their side effects, including increased body weight and liver fat, have restricted their use (Ahmadian *et al.*, 2013). It is therefore necessary to find a new PPARγ-specific ligand that avoids these side effects while retaining the insulin-sensitizing role. PPARγ is the master regulatory gene for adipocyte formation, and Pparγ knockout mice died embryonically (Gregoire *et al.*, 1998). Rosiglitazone is a full agonist of PPARγ and widely used as an inducer of adipogenesis, so it easily leads to body weight increase in mice. Recently, SR11664 was identified as a new partial agonist of PPARγ that has no effect on adipogenesis, yet still exerts an anti-diabetic effect on obese mice without many unwanted side effects (Choi *et al.*, 2011). Telmisartan, a drug marketed for hypertension, has been validated as a partial agonist of PPARγ, but it decreases body weight gain, unlike rosiglitazone (Araki *et al.*, 2006). In addition, many preclinical partial agonists of PPARγ not only have limited adipogenic ability *in vitro* but also resist body weight increase *in vivo*, such as nTZDpa, KR-62980, and halofenate (Zhang *et al.*, 2007). Interestingly, these poorly known agonists showed little effect on thermogenesis (Ohno *et al.*, 2012). In the present study, we found that the natural product formononetin is also a partial agonist of PPARγ and promotes thermogenesis both *in vivo* and *in vitro*. Another partial agonist of PPARγ, calycosin, which has a structure analogous to formononetin,

showed no effect on the expression of *Ucp1*. Consistent with the previous study, as a partial agonist, formononetin had poor adipogenic capacity and did not promote body weight increase. In addition, liver weight and TG content in mice treated with formononetin showed no significant difference from that of control mice, excluding the fatty liver side effect of rosiglitazone (Figure S9). It is noteworthy that full agonists of PPARγ such as rosiglitazone increases the protein expression of UCP1 in adipocytes both *in vitro* and *in vivo*, but was found to have no effect on energy expenditure in vivo (Sell *et al*., 2004). Sell and colleagues studied2-(2-[4-phenoxy-2-propylphenoxy]ethyl)indole-5-acetic acid or COOH, a full PPARγ agonist with similar structure to rosiglitazone, and reported that COOH strongly increased protein levels of UCP1 in both BAT and WAT in wild type C57BL/6J and ob/ob obese mice without apparent enhancing energy expenditure. In contrast, formononetin is clearly shown to up-regulate UCP1 protein and increase energy expenditure while a partial PPARγ agonist calycosin does neither, therefore, different PPARγ ligands can induce a spectrum of response (Hughes *et al*., 2012). Although the detailed molecular mechanism was not clear, it is possible that expression of additional genes and/or different levels of the induced genes by modulation of PPARγ activity play critical roles in functional thermogenesis mediated by activated UCP1 protein. Indeed, Fmoc-L-Leu, a partial PPARγ agonist with about 100 to 1000 times less potency compared to rosigliazone, has similar capacity to induce UCP1 expression to formononetin and reduced adipogenic potential as does formononetin (Rocchi *et al*., 2001; Mao *et al*., 2017). Formononetin is unlikely to affect the β3 adrenoceptor signaling pathway based on the following. First of all, formononetin had synergistic effects when β3 adrenoceptor agonist CL316243 was added (Figure 3A). And secondly, formononetin increased expression of UCP1 but did not work to reduce body weight gain under thermoneutrality (Figure S5), suggesting a sympathetic activation is essential for its activity. However, more clinical

experiments are obviously needed to determine whether formononetin is a suitable medicinal compound for the treatment of obesity and diabetes in patients. Nonetheless, our study pointed out a possible research direction to explore in that formononetin might serve as a potential drug candidate as a PPARγ partial agonist due to its beneficial effect on obesity and insulin resistance.

Astragalus membranaceus is a traditional Chinese herbal medicine that has been widely used with almost no harm to human health; more importantly, it is commonly used as a daily herbal supplement to maintain health in China. For example, it is mostly used to make soup with chicken, Chinese dates (*Ormosia hosiei*), or other foods (Song *et al.*, 2014). To elucidate its molecular mechanisms in human health, the functions of its various constituents have been studied, including polysaccharides, saponins, and flavonoids. Among these, the astragalus polysaccharides have been most studied and demonstrated to be beneficial for insulin resistance, cardiac hypertrophy, and so on (Liu *et al.*, 2014; Luan *et al.*, 2015). Astragaloside IV (a saponin) has been reported to regulate adiponectin secretion and improve insulin resistance in obese mice (Xu *et al.*, 2009). However, the roles of the other constituents remain poorly characterized. In this report, a new role of *Astragalus membranaceus* and its constituent formononetin in adipocyte thermogenesis by modulating PPARγ activity has been revealed. Our study supports the belief that *Astragalus membranaceus* and its constituent formononetin support health and reduce obesity.

Acknowleagements

This study was supported in part by National Basic Research Program of China (2016YFC1305000 and 2010CB945500), the strategic priority program on development of new drug of the Chinese Academy of Sciences (XDA12040325), Natural Science Foundation of China (81700742, 81400825,

81774134, 81327801 and 31301019), a key international collaborative fund from the Chinese Academy of Sciences (154144KYSB20150019), Natural Science Foundation of Guangdong province (2016A030310122), International collaborative funds from Guangdong Province (2013B051000090 and 2015A050502041), Science and Technology Planning Project of Guangdong Province (2017B030314056) and Guangdong Provincial Public interest research and capacity building projects (2014A010107024).

Author Contributions

T.N., S.Z., L.M., Y.Y., W.S., X.L., S.L., K.L. and Y.S. performed the experiments. T.N., Z.Z., A.X., C.W.M., P. L., S.L., Y.X., C.W., P. D. and D.W. analyzed and interpreted the data. T.N. and D.W. conceived and designed the experiments. T.N., X.H. and D.W. wrote and edited the manuscript. D.W. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

The authors declare no competing financial interests.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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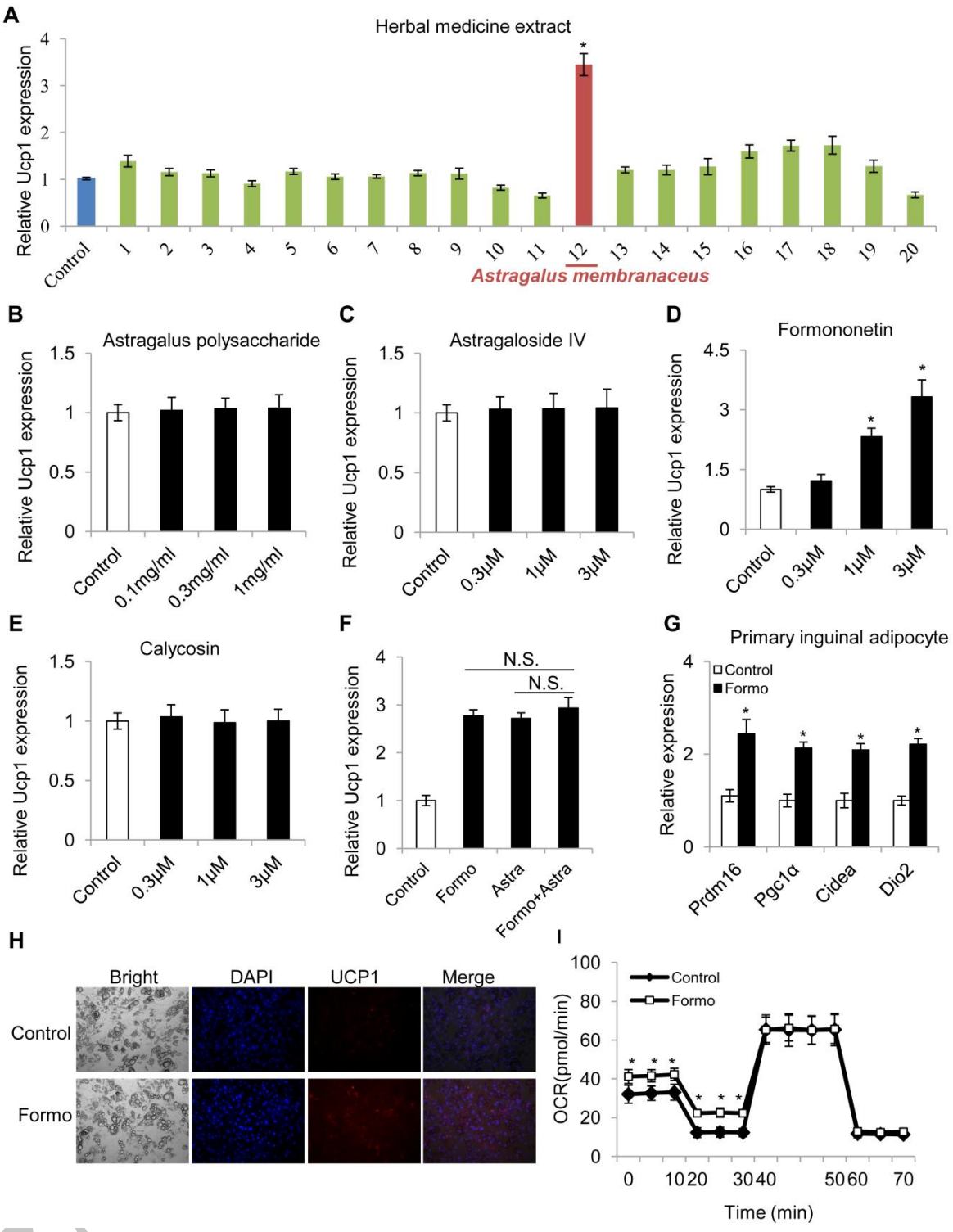

Figure 1. Formononetin derived from *Astragalus membranaceus* extract induced adipocyte thermogenesis *in vitro*.

Inguinal adipose stromal cells were differentiated into adipocytes, and then treated with a Chinese herbal medicine extract at a ratio of 1:1,000 for 24 h, and harvested for qPCR. (A) *Ucp1* mRNA

expression in primary inguinal adipocytes by various Chinese herbal medicine extracts (n = 5): 1.*Cichorium intybus* (1.5 mg/ml), 2.*Cinnamomum cassia* (0.3 mg/ml), 3.*Coix lachryma-jobi* (1.0 mg/ml), 4.*Zingiber officinale* (1 mg/ml), 5.*Eucommia ulmoides* (1.2 mg/ml), 6.*Eugennia caryophyllata* (0.2 mg/ml), 7.*Sophora japonica* (0.8 mg/ml), 8.*Hippophae rhamnoides* (0.42 mg/ml), 9.*Alpinia officinarum* (0.5 mg/ml), 10.*Lonicera japonica* (1 mg/ml), 11.*Morus alba* (1.2 mg/ml), 12.*Astragalus membranaceus* (2 mg/ml), 13.*Atractylodes lancea* (0.6 g/ml), 14.*Lycium barbarum* (1.2 mg/ml), 15.*Piper nigrum* (0.1 mg/ml), 16.*Sterculia lychnophora* (1 mg/ml), 17.*Morus alba* (0.8 mg/ml), 18.*Citrus reticulata* (0.6 mg/ml), 19.*Euodia rutaecarpa* (0.3 mg/ml), and 20.*Trigonella foenum-graecum* (0.8 mg/ml). (B-E): Effects of constituents (B) astragalus polysaccharide, (C) astragaloside IV, (D) fomononetin, (E) calycosin of *Astragalus membranaceus* on *Ucp1* expression in primary differentiated inguinal adipocytes were analyzed by qPCR(n = 5). (F) The synergistic effect on *Ucp1* expression of *Astragalus membranaceus* extract and formononetin (n = 5). (G) Expression of thermogenic genes in primary inguinal adipocytes (n = 5). For quantitative RT-PCR experiments**,** 18S ribosomal RNA was adopted as an internal standard to control for unwanted sources of variation. The average mRNA levels of thermogenic genes were normalized to the blank control (baseline). (H) Immunofluorescent staining of adipocytes treated with DMSO (control) or formononetin. (G) OCR in primary inguinal adipocytes treated with DMSO (control) or formononetin (n = 5 well/group). Data represent mean ± SEM, the experiments had been repeated three times, *$p<0.05$.

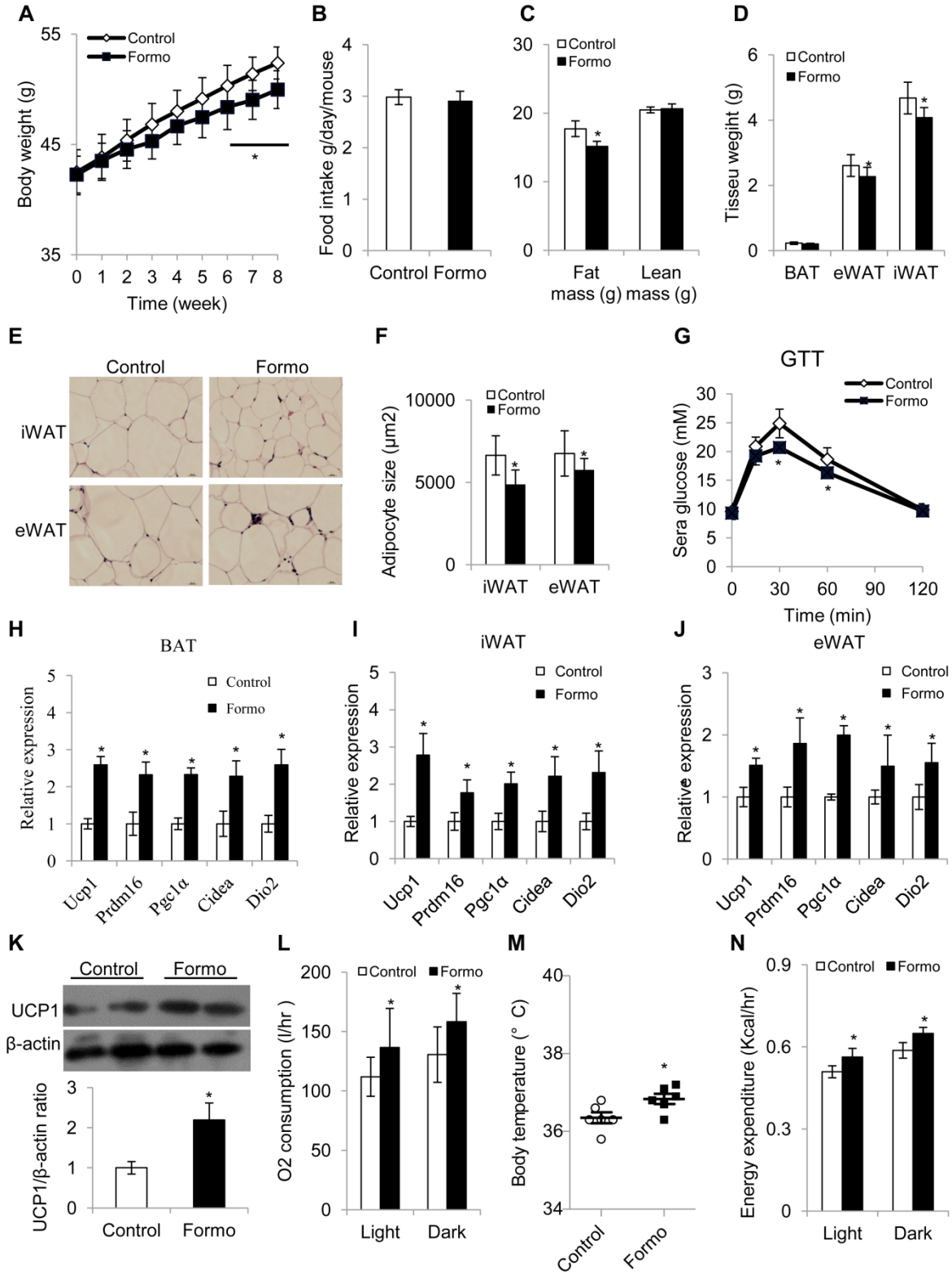

Figure 2. DIO mice treated with formononetin resisted diet-induced obesity.

(A-D): Eight-week-old C57BL/6J male mice (n = 10 mice) were fed a high fat diet for 8 weeks to become obese and then treated with the vehicle (control) or formononetin for 8 weeks. Effects on (A)

food intake, (B) bodyweight, (C) body composition, and (D) tissue weight. (E-F): Representative H&E staining of epididymal white adipose tissue (eWAT) and inguinal white adipose tissue (iWAT). (G): Glucose tolerance test (n = 10). (H-J): Quantitative PCR analysis of thermogenic genes in (H) iWAT, (I) eWAT, and (J) BAT from mice treated with the vehicle (control) or with formononetin for 8 weeks (n = 5). For quantitative RT-PCR experiments, 18S ribosomal RNA was adopted as an internal standard to control for unwanted sources of variation. The average mRNA levels of thermogenic genes were normalized to the control mice (baseline). (K) Western blot analysis of UCP1 in BAT (above) and densitometric analysis of the relative abundance of UCP1 (below) (n = 5), β-actin was adopted as an internal standard to control for unwanted sources of variation, the ratio of UCP-1 expression in BAT was normalized to that in control mice (baseline). (L) Oxygen consumption (n = 5). (M) Body temperature (n = 5). (N) Energy expenditure (n = 5). Vehicle was used as control. Data represent mean ± SEM, the experiments had been repeated three times, *$p<0.05$.

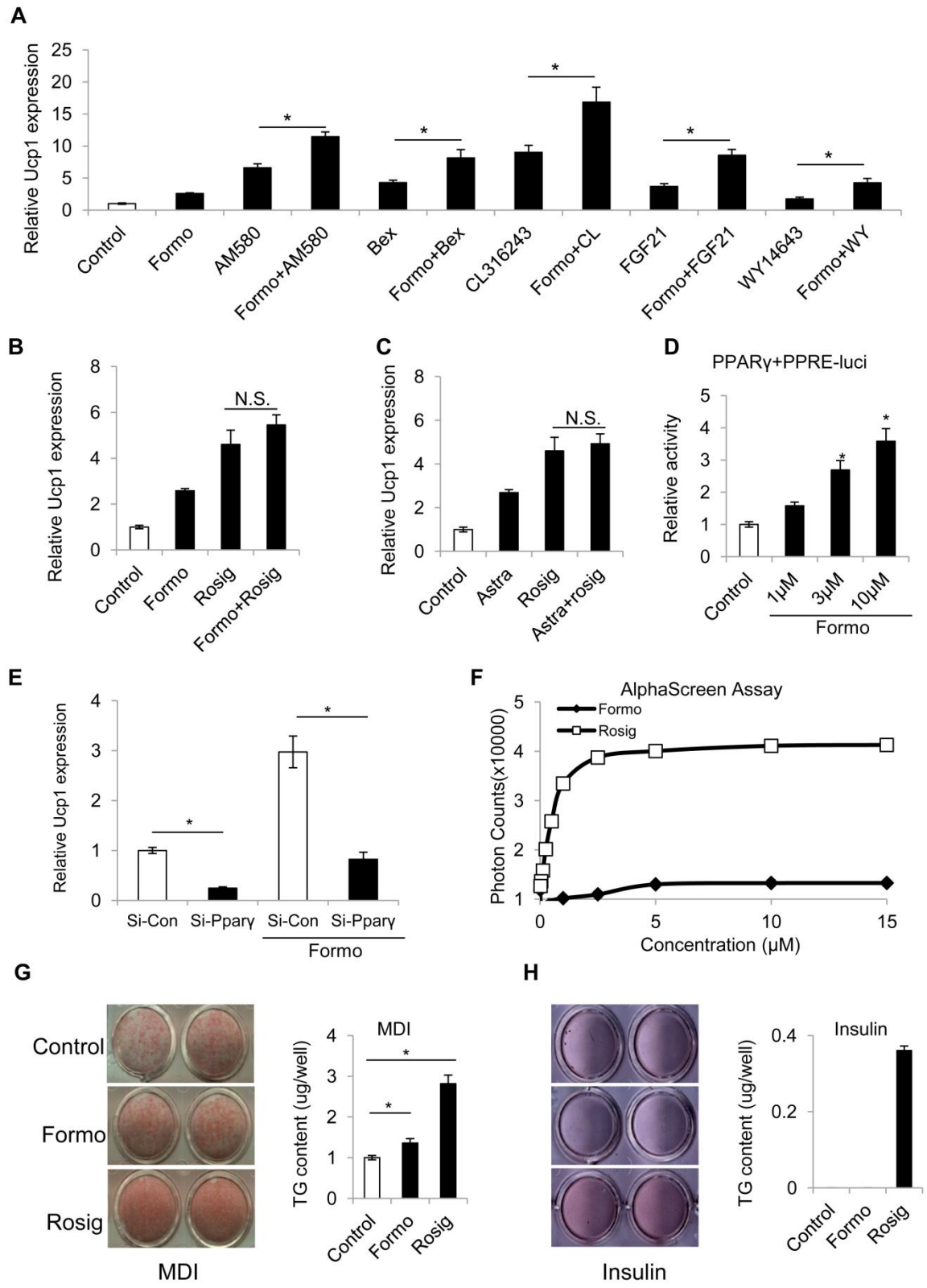

Figure 3. PPARγ mediates formononetin function.

(A-C): Synergistic effect on *Ucp1* expression in differentiated inguinal adipocytes of known adipocyte thermogenesis agonist with (A) formononetin, rosiglitazone with (B) formononetin and (C)

Astragalus membranaceus extract (n = 5). For quantitative RT-PCR experiments, 18S ribosomal RNA was adopted as an internal standard to control for unwanted sources of variation. The average mRNA expression of *Ucp1* was normalized to the blank control (baseline). (D) Formononetin dose-dependently promoted luciferase activity in COS7 cells transfected with PPARγ and PPRE-luciferase plasmids (n = 5). The luciferase activity was normalized to the blank control (baseline). (E) SiRNA knockdown of *Pparγ* impaired adipocyte thermogenesis induced by formononetin (n = 5). 18S ribosomal RNA was adopted as an internal standard to control for unwanted sources of variation. The average mRNA expression of *Ucp1* was normalized to the si-RNA control as baseline. (F) Dose-response curves of the PPARγ LBD to PGC-1α-1 in the presence of rosiglitazone and formononetin (n = 5). (G-H): Oil red staining (left) and quantitation of TG contents (right) of 3T3-L1 differentiated cells with the vehicle (control), rosiglitazone, or formononetin under (G) MDI cocktail and (H) insulin only (n = 5). Data represent mean ± SEM, the experiments had been repeated three times, *$p<0.05$.

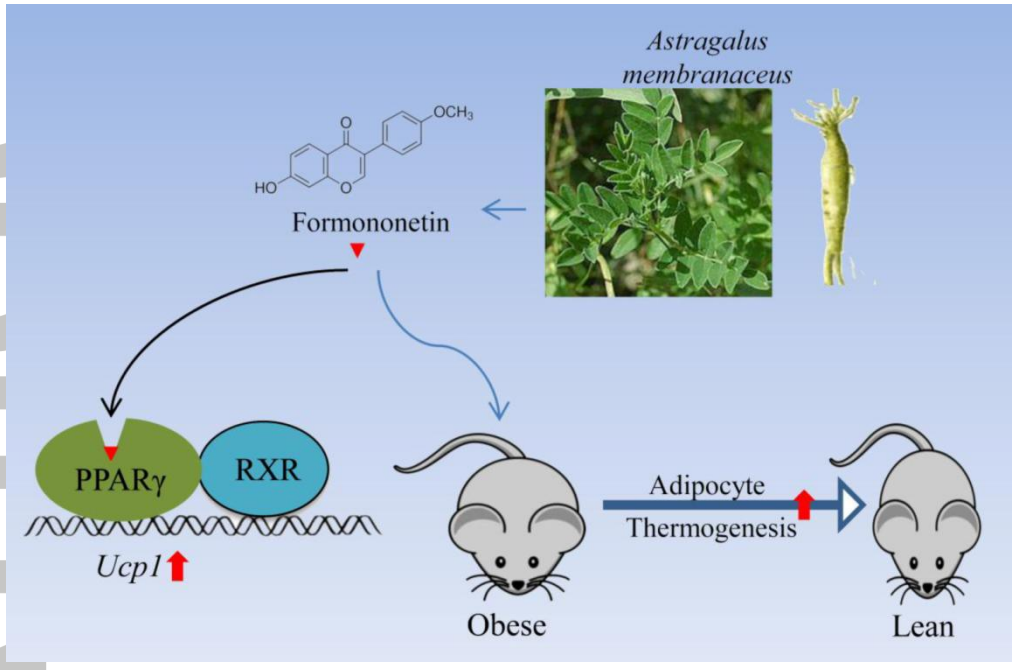

Figure 4. Schematic diagram of the adipocyte thermogenesis mechanism by formononetin and *Astragalus membranaceus*.

Astragalus membranaceus and its constituent formononetin resist high fat diets induced obesity by promoting adipocyte thermogenesis. Formononetin binds with PPARγ to form a heterodimer with RXR to drive expression of *Ucp1* in adipocytes.