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Lycium barbarum (Goji Berry) extracts and its taurine component inhibit PPAR-γ-dependent gene transcription in human retinal pigment epithelial cells: Possible implications for diabetic retinopathy treatment

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ABSTRACT

The peroxisome proliferator activated receptor-γ (PPAR-γ) is involved in the pathogenesis of diabetic retinopathy. Diabetic retinopathy is a preventable microvascular diabetic complication that damages human retinal pigment epithelial cells. Taurine is abundant in the fruit of *Lycium barbarum* (Goji Berry), and is reportedly beneficial for diabetic retinopathy. However, the mechanism of its action is unknown. Hence, we have investigated the mechanism of action of an extract from *L. barbarum* on a model of diabetic retinopathy, the retinal ARPE-19 cell line, and identified the receptor function of taurine, an active component of *L. barbarum* (Goji Berry) extract, which is potentially responsible for the protective effect on diabetic retinopathy. We demonstrate for the first time that *L. barbarum* extract and its taurine component dose-dependently enhance PPAR-γ luciferase activity in HEK293 cell line transfected with PPAR-γ reporter gene. This activity was significantly decreased by a selective PPAR-γ antagonist GW9662. Moreover, *L. barbarum* extract and taurine dose-dependently enhanced the expression of PPAR-γ mRNA and protein. In an inflammation model where ARPE-19 cells were exposed to high glucose *L. barbarum* extract and taurine down-regulated the mRNA of pro-inflammatory mediators encoding MMP-9, fibronectin and the protein expression of COX-2 and iNOS proteins. The predicted binding mode of taurine in the PPAR-γ ligand binding site mimics key electrostatic interactions seen with known PPAR-γ agonists. We conclude that PPAR-γ activation by *L. barbarum* extract is associated with its taurine content and may explain at least in part its use in diabetic retinopathy progression.

1. Introduction

The peroxisome proliferator activated receptor-γ (PPAR-γ) is a ligand-inducible transcription factor that belongs to a large superfamily comprising the nuclear receptors for steroids, thyroid hormones, and retinoids [1]. PPAR-γ plays an important role in adipogenesis, glucose metabolism, angiogenesis and inflammation [2,3]. Available synthetic PPAR-γ ligands from the thiazolidinedione family are rosiglitazone (RG), pioglitazone and troglitazone, whilst, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PG) is a potent natural PPAR-γ ligand [4–6]. PPAR-γ is heterogeneously expressed in the mammalian eye, prominently in the retinal pigmented epithelium, photoreceptor outer segments and choriocapillaries [7–9]. Recent studies have shown that retinal expression of PPAR-γ was suppressed in experimental models of diabetes and in endothelial cells treated with high glucose [10]. Moreover, PPAR-γ ligands are potent inhibitors of corneal angiogenesis and neovascularization [11,12]. These findings support the involvement of PPAR-γ in the pathogenesis of diabetic retinopathy (DR). Moreover, the synthetic PPAR-γ activator RG has been shown to delay the onset of DR [13].

DR is one of the most common microvascular complications of diabetes and a leading cause of preventable blindness worldwide [14]. Nearly 80% of patients develop DR after two decades of diabetes [15]. Early detection and prevention are the current management strategy for patients with DR [16]. The chronic hyperglycemia of diabetes results in damage to all major cells of the retina, such as vascular cells (endothelial cells and pericytes) [17,18] and pigment epithelial cells [19] and is a major factor responsible for DR. The retinal pigment epithelial (RPE) cells form a monolayer between the neuroretina and the choriocapillaris, which are the essential components of the outer blood retinal barrier (BRB) that maintain physiological and structural balance within the retina [20,21]. In response to damage caused by the

Abbreviations: PPAR-γ, peroxisome proliferator activated receptor-γ; LB, fruit from *Lycium barbarum*; RT-PCR, reverse transcription-polymerase chain reaction; HEK, human embryonic kidney; ARPE-19, human retinal epithelial cell line; DR, diabetic retinopathy; RG, rosiglitazone; PG, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2.

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hyperglycemic condition, RPE cells migrate and proliferate, leading to a breakdown in adhesion between the RPE and the choroidal capillaries, followed by BRB breakdown compromising blood flow within the RPE layer, resulting in eventual retinal edema [22]. These cascade episodes trigger the serum components and inflammatory cells to enter the vitreous cavity and sub-retinal space, exposing the RPE cells to a variety of cytokines, pro-inflammatory mediators, extracellular matrix proteins and growth factors, causing DR [23]. Moreover, studies have shown that up-regulation of pro-inflammatory mediators, such as matrix metalloproteinase-9 (MMP-9), fibronectin, cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) play an important role in the development of DR [24,25].

Fruit from *Lycium barbarum* (LB) in the family Solanaceae is a well-known traditional Chinese medicine [26]. LB has been widely used as nutritional food product with a large variety of beneficial effects, such as reducing blood glucose and serum lipids, anti-oxidant, immune-modulation, neuroprotection, and anti-inflammatory activity [27–29]. There is a growing body of evidence indicating that LB intake increases the fasting plasma zeaxathin levels, beneficial for maintaining macular pigment density in age-related macular degeneration [30]. Moreover, LB has been shown to be effective in the treatment of glaucoma and modulating immunity in retinal ganglion cells in a rat hypertension model [31,32]. However, the mechanism of the beneficial effect of LB in DR has not been studied. LB contains 18 types of amino acids, including taurine, a non-essential free amino acid, which is one of the chemical components abundantly present in LB [33,34]. Several previous articles have reported that taurine potentiates the effect of insulin [35,36] and possibly modulates the insulin receptor [37,38]. Moreover, taurine has been recommended as a complementary therapeutic agent for the prevention of diabetic complications in type II diabetes [39,40].

Despite the growing body of evidence both from *in vitro* and animal research for LB in various medical applications in recent years, there is limited research available establishing its efficacy and mode of action in modulating eye related pathology. Moreover, the taurine component of LB needs to be further investigated through molecular biology to pin-point its therapeutic potential for the treatment of DR. Therefore, the aim of this study is to investigate the mechanism of action of the extract from LB on DR and its receptor function in a retinal cell line model. Our study has identified taurine as one of the major components of LB extract responsible for the activity of PPAR-γ through screening of crude extracts of LB (water, ethanol and methanol) using molecular bioassay and phytochemical analysis methods.

2. Methods

2.1. Fractionation and taurine analysis of LB extracted with different solvents

Dried LB was purchased in raw powder form (batch no. 53101: DeDu Holdings, West Ryde, Australia). The extraction of LB was performed as previously described [41] with modifications. Briefly, 250 mg of fine LB powder was extracted four times with 2.5 ml of methanol by sonication at room temperature for 15 min, followed by centrifugation for 5 min; the same extraction procedure was carried out with methanol/water (1:1), ethanol (100%) and water. The solvents were then evaporated under reduced pressure below 50 °C to give the extracts. The identification and quantification of taurine in the LB extracts was undertaken by a thin layer chromatography (TLC) densitometry method as previously described, with some modifications [42,43]. Briefly, standard solutions of taurine were applied to a silica gel plate, and following elution with mobile phase the levels recorded as area units by densitometry. Calibration curves were prepared by plotting area unit vs. concentration of taurine applied. The amount of taurine in the LB samples was calculated using the calibration curve. The percentages of taurine content in LB extracts were then compared.

2.2. Tissue culture and treatment

The human retinal epithelial cell line, ARPE-19 was provided by Dr. Weiyong Shen (Save Sight Institute, Sydney, Australia). Cells were cultured as previously described [44]. Briefly, the cells were cultured in a humidified incubator at 37 °C in 5% CO_2 in 10% fetal bovine serum–defined minimal essential medium (FBS–DMEM)-F12 medium containing 5.5 mM D-glucose, supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen, Mulgrave, Australia). The culture medium was replaced with fresh medium every second day. Upon confluence, cultures were passaged by dissociation in 0.05% (w/v) trypsin (Gibco-Life Technologies, Roseville, MD, USA) in phosphate buffered saline (PBS) pH 7.4. For high glucose-induced functional studies, cells were maintained in fresh medium containing 1% FBS for 2 h prior to use in the experiments. Cells were then treated with samples, RG, PG or vehicle (0.5% DMSO) for 6 h. The treatment with extract and drug samples was continued for 48 h during the incubation in normal (5.5 mM) or high (33.3 mM) glucose-D. The cells incubated in 27.5 mM mannitol (M) served as osmotic control [45]. Human embryonic kidney (HEK) 293-cell line was obtained from American Type Culture Collection. The cells were grown in DMEM/F-12, as previously described [46]. Briefly, the HEK293 cells were cultured in DMEM-F12 medium consisting of 10% fetal bovine serum, 1% penicillin/streptomycin solution. Cytotoxicity of LB extract and taurine used in the experiments were examined by MTS assay (Promega, Alexandria, Australia) as previously described [47] and in all conditions treatment showed little or no effect on cell viability (>90% viability remained) (data not shown).

2.3. Transfection and luciferase analysis of PPAR-γ luciferase activity

To determine PPAR-γ reporter gene luciferase activity, the standard cell type regularly used for transfection studies, were transfected as previously described [46,48]. The plasmids used for transfection were tK-PPREx3-Luc plasmid (a kind gift from Dr. Teruo Kawada, Kyoto University, Japan); pSG5-hPPAR-γ1 plasmid (~1/5 kb) (a kind gift of Dr. Willa Hsueh, University of California, Los Angeles) and pSV-β-galactosidase control vector (Promega, Alexandria, Australia) to normalize transfection efficiencies.

Cells were transfected with FuGENE6 transfection reagent (Roche, Castle Hill, Australia) in accordance with the manufacturer's instructions. At 48 h before transfection, HEK293 cell line was seeded at 5×10^5 cells/T25 flask in 5 ml of Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and supplemented with 1% penicillin and streptomycin, 1% L-glutamine, 20 mM HEPES [46,48]. After 48 h, the cells were then harvested and plated into 96-well plates at 3×10^4 cells per well in complete transfection media and allowed to attach for 6 h. The cells were then treated with the PPAR-γ positive control (rosiglitazone), LB or vehicle (0.5% DMSO). After 48 h, the cells were lysed and assayed for luciferase and β-galactosidase activities using the Bright-Glo Luciferase Assay System and Beta-Glo Assay System (Promega, Alexandria, Australia), respectively. GW9226, a selective PPAR-γ antagonist was added 1 h before addition of rosiglitazone and test samples. There results were expressed as relative luciferase activity (fold difference compared to negative control). To determine the selectivity of receptors for LB, RG was used as the positive control for PPAR-γ.

2.4. Gene expression

Gene expression was determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), as described previously [49]. Briefly, total mRNA from ARPE-19 cells using TRIzol reagent was converted to single-stranded cDNA using superscript II Rnase H reverse transcriptase as per the manufacturer's instruction (Invitrogen, Mulgrave, Australia). For semiquantitative PCR, 0.5 μg of cDNA template was added to 20 μM of forward and reverse primer and 1 unit of Platinum® Pfx DNA polymerase (Invitrogen, Mulgrave, Australia). PCR was performed on a thermocycler, PTC-200 DNA engine (MJ Research Inc., USA). The genes examined were PPAR-γ (L40904; 382 bp; sense: 5′-GAGCCCAAGTTTGAGTTTGC-3′ and antisense: 5′-TGGAAGAAGGGAAATGTTGG-3′), fibronectin (NM002026; 438 bp; sense: 5′-TCCACAAGCGTCATGAAGAG-3′ and antisense: 5′-ATACCACACCAGGCTTCAGG-3′), MMP-9 (NM004994.2; 179 bp; sense: 5′-TTGACAGCGACAAGAAGTGG-3′ and antisense: 5′-GCCATTCACGTCGTCCTTAT-3′) and β-actin (NM001101; 234 bp; sense: 5′GGACTTCGAGCAAGAGATGG-3′ and antisense: 5′-AGCACTGTGTTGGCGTACAG-3′). After the PCR amplification, the PCR samples were electrophoresed on 1.2% agarose gels at 70 V and stained with ethidium bromide (10 μg/ml) and photographed on top of a 280 nm UV light box (Biorad® Gel Doc 100). The gel images were digitally captured with a CCD camera and analyzed with ImageJ 1.29x (NIH, USA). RT-PCR values are presented as a ratio of the specified gene signal in the selected linear amplification cycle divided by the β-actin signal. To determine the selectivity of receptors for LB, RG and PG were used as the positive control for PPAR-γ.

2.5. Protein extraction and semi-quantitative western blotting analysis

Immunoblots were conducted as described previously [50]. The proteins from the cells were prepared using the Ripa lysis buffer (25 mM Tris buffer (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). The lysed cells were centrifuged at 12,000 rpm (Micromax RF centrifuge, Thermo IEC, MA, USA) for 10 min and supernatants resolved by SDS-PAGE, 4–12% (Invitrogen, Mulgrave, Australia). Protein was transferred to cellulose membrane with transfer buffer (Tris base 25 mM, glycine 192 mM, pH 8.3) and blocked in 5% skim milk powder (Sigma–Aldrich St. Louis, MO, USA) overnight. The primary antibodies (Santa Cruz Biotechnology, USA) were anti-PPAR-γ mouse monoclonal primary antibody (1:500 dilution), anti-iNOS mouse monoclonal primary antibody (1:100 dilution) and anti-COX-2 goat polyclonal primary antibody (1:500 dilution). After incubation with the primary antibody for 1 h at room temperature, the membrane was washed and further incubated with horseradish peroxidase conjugated anti-mouse secondary antibodies (1:6000 dilution; Santa Cruz Biotechnology, USA). Bound antibodies were detected using enhanced chemiluminescence with Lumi-Light Western Blotting Substrate (Roche, Castle Hill, Australia). The membranes were exposed to X-ray film (Kodak, USA) and developed using the SRX-101A X-ray developer (Konica, Taiwan). The resultant films were quantified by scanning densitometry using ImageJ (National Institutes of Health, Bethesda, MD). Protein expression was quantified by normalization to α-tublin. The membranes were re-probed with anti-α-tublin primary antibody (1:10,000 dilution; Santa Cruz Biotechnology, USA) after stripping and overnight blotting with 5% skim milk. The membranes were re-incubated with horseradish peroxidase conjugated anti-mouse secondary antibody and detected using the same procedure as described above. Cell lysate protein concentrations were determined by the BCA (bicinchoninic acid) assay (Thermo Fischer Scientific, Scoresby, Australia) according to the manufacturer's instructions.

2.6. Statistical analysis

All results are expressed as means ± SEM. Data was analyzed by 1-factor analysis of variance (ANOVA). If a statistically significant effect was found, the Newman–Keuls test was performed to isolate the difference between the groups. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

2.7. Chemicals

Rosiglitazone was purchased from Alexis Biochemicals (San Diego, Calif, USA). GW9662, pure taurine compound, mannitol, glucose-D, Tris base and glycine were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.8. Computer modelling—receptor preparation and induced-fit docking

Coordinates for PPAR-γ receptors 1FM9 and 1FM6 were taken from the RCSB protein data bank (PDB) [51] and prepared using the Protein Preparation Wizard, which is part of the Maestro software package [52]. Bond orders and formal charges were added for heterogroups, and hydrogens were added to all atoms in the system. To optimise the hydrogen bond network, His tautomers and ionization states were predicted, 180° rotations of the terminal χ angle of Asn, Gln, and His residues were assigned, and hydroxyl and thiol hydrogens were sampled. Water molecules in all structures were removed. For each structure, a brief relaxation was performed using an all-atom constrained minimization carried out with the impact refinement module (Impref) [53] using the OPLS-2005 force field to alleviate steric clashes that may exist in the original PDB structures. The minimization was terminated when the energy converged or the rmsd reached a maximum cut off of 0.30 $Å^3$.

The structure of taurine was built in Maestro [52] and docked flexibly into the ligand-binding site of the prepared 1FM9 and 1FM6 receptors using the induced-fit docking (IFD) protocol [54–56]. Briefly, the IFD protocol begins by flexibly docking taurine with Glide [55] into the PPAR-γ receptor. The binding site was defined by a rectangular box surrounding the X-ray ligands: farglitazar and rosiglitazone, observed in complex with PPAR-γ as part of the PPAR-γ/RXR-α heterodimer crystal structure (1FM9 and 1FM6, respectively) [57]. To generate a diverse ensemble of ligand poses, the procedure uses reduced van der Waals radii and an increased Coulomb–vdW cutoff. For each resulting pose, a Prime structure prediction [56] is then used to accommodate taurine by reorienting side chains within 5 $Å^3$ of the taurine poses. These residues plus taurine are then minimized with the OPLS-2005 force field. Finally, each pose is re-docked flexibly into its corresponding low energy protein structures and the resulting complexes are ranked according to Glide score [55]. The top five complexes as determined by IFD score [54] from each of the runs on 1FM9 and 1FM6 were then selected to give a combined set of ten final poses.

3. Results

3.1. Screening of crude extracts of LB on PPAR-γ activity in HEK293 cell line and taurine contents

As PPAR-γ plays an important role in delaying the progression of DR, we investigated the effects of LB extracts on PPAR-γ luciferase activity *in vitro*. The methanol extract was found to produce the highest PPAR-γ activation (3.37-fold), followed by smaller non-significant effects with methanol/water and water extracts, with only minor activation by the ethanol extract (Fig. 1a).

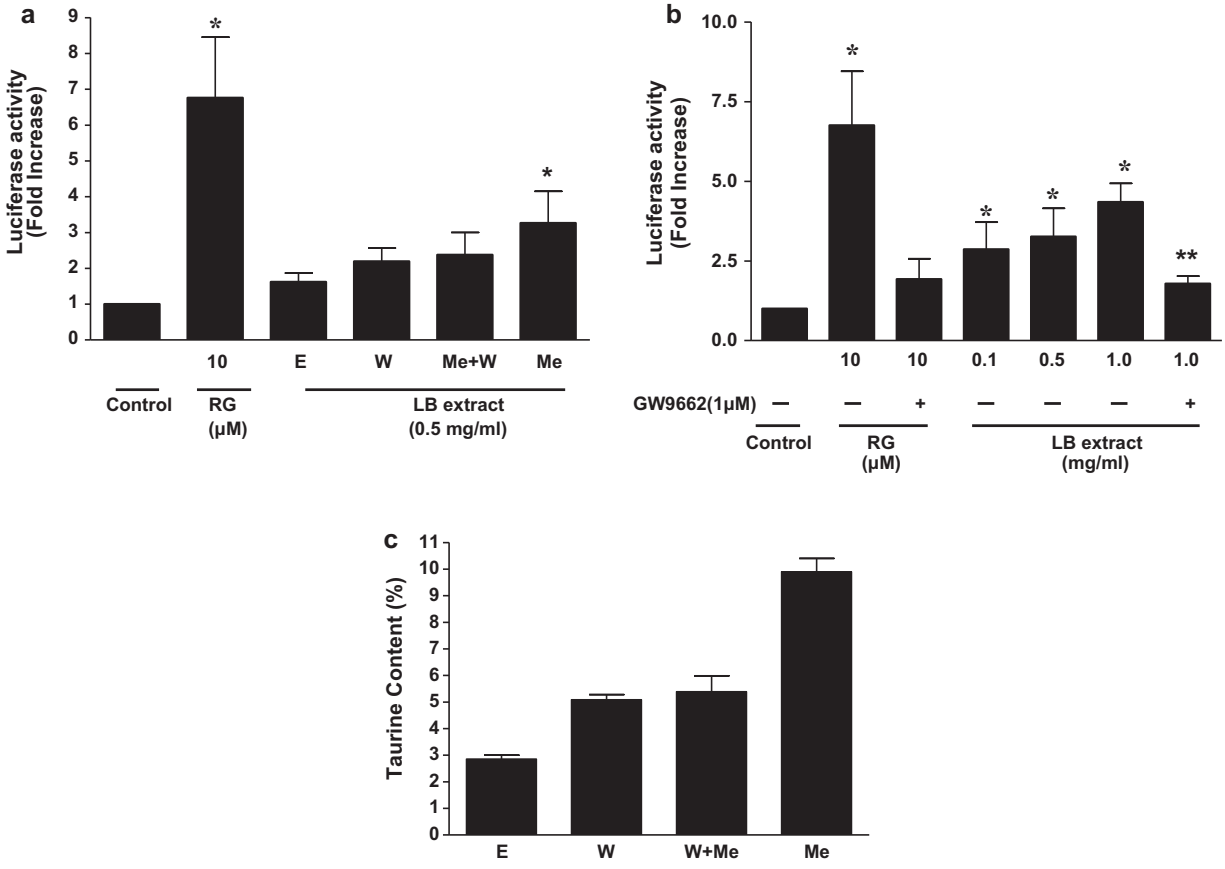

Fig. 1. Screening of crude extracts of LB on PPAR-γ activity in HEK293 cells and taurine contents. RG (10 μM) was used as a positive control. Levels in the control were arbitrarily assigned a value of 1.0. (a) Fold activation of PPAR-γ luciferase activity in HEK293 cells transfected with reporter genes by four LB extracts (0.5 mg/ml): methanol (Me), water (W), methanol/water (Me + W) and ethanol (E). (b) The most active LB methanol extract dose-dependently enhanced PPAR-γ luciferase activity, which was significantly suppressed by a selective PPAR-γ antagonist GW9226 (1 μM). Values are means ± SEM. ($n = 4$, each in triplicate). $^{*}P < 0.05$ versus Control, $^{**}P < 0.01$ versus LB extract (1 mg/ml) without GW9662. (c) taurine content (%) in methanol (Me), water (W), methanol/water (Me + W) and ethanol (E). Values from TLC densitometry are means ± SD ($n = 4$).

The most active methanol extract was examined for dose-dependency. The results show that LB extract (0.1, 0.5 and 1 mg/ml) concentration-dependently enhanced PPAR-γ luciferase activity in the HEK293 cell line (2.87-, 3.37-, and 4.33-fold, respectively) (Fig. 1b). The increase was significantly blocked (41.3%) by a selective PPAR-γ antagonist GW9226. As our results (see below) indicated that the effects of the methanol extract were mimicked by taurine, a known active component of LB, the content of taurine was examined in each LB fraction. The activation of PPAR-γ correlated with the content of taurine in the extracts, with the methanol fraction containing the highest concentration (10.7 ± 1.1%), followed by methanol/water (1:1 v/v) (5.3 ± 0.7%) and water (5.0 ± 0.6%), whilst the ethanol fraction had lower contents (3.0 ± 0.4%) (Fig. 1c).

3.2. Effects of LB extract on PPAR-γ mRNA and protein expression

To further understand the mechanism of LB extract in DR through the PPPAR-γ, we investigated the effect of LB extract on PPAR-γ protein and mRNA expression in the ARPE-19 cell line. RT-PCR analysis showed that LB extract increased PPAR-γ mRNA expression (Fig. 2a) in a dose dependent manner (by 3.13-, 3.66- and 5.06-fold, respectively). Moreover, Western blotting analysis demonstrated that LB extract enhanced PPAR-γ protein expression (Fig. 2b) in a dose dependent manner (by 1.84-, 2.87- and 3.78-fold, respectively).

3.3. LB extract down-regulates gene and protein expression of pro-inflammatory mediators in high glucose-treated ARPE-19 cells

Inflammation is a major part of the pathogenesis of DR, and it has been suggested that PPAR-γ ligands exert therapeutic effects as modulators of inflammation [58]. To determine whether the beneficial effect of LB extract (0.1 mg/ml, 0.5 mg/ml and 0.75 mg/ml) in an inflammation model of DR might be attributed to PPAR-γ-induced anti-inflammatory activity, we first examined the mRNA and protein expressions of pro-inflammatory mediators including MMP-9, fibronectin, iNOS and COX-2 in high glucose-induced ARPE-19 cells. The results showed that LB extract dose-dependently down-regulated the expression of inflammatory mRNAs encoding MMP-9 (by 6.38-, 4.81- and 3.90- fold, respectively) (Fig. 3a) and fibronectin (by 5.96-, 3.88- and 3.01-fold, respectively) (Fig. 3b). LB extract dose-dependently down-regulated the protein expression of pro-inflammatory mediators encoding COX-2 (by 6.07-, 4.30- and 2.52-fold, respectively) (Fig. 3c) and iNOS (by 7.01-, 5.70- and 2.93-fold, respectively) (Fig. 3d).

3.4. Effects of taurine on PPAR-γ activity

To confirm that taurine in the LB extract is active in induction of PPAR-γ, we tested the dose dependency of the effect of taurine on PPAR-γ luciferase activity in the cell lines. The results showed that

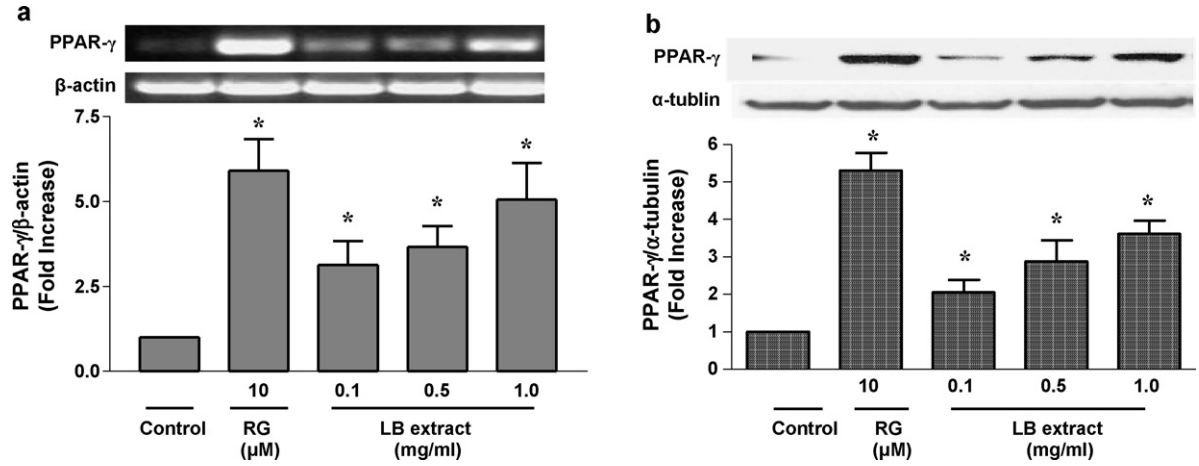

Fig. 2. Effects of LB methanol extract on PPAR-γ activity. (a) mRNA and (b) protein expression of PPAR-γ in ARPE-19 cells treated with the LB methanol extract (0.1, 0.5 and 1 mg/ml) and RG (10 μM). The results were normalised to β-actin (for mRNA) or α-tublin (for protein). Control levels were arbitrarily assigned a value of 1.0. All values are means ± SEM ($n = 6$). $^{*}P < 0.05$ versus control.

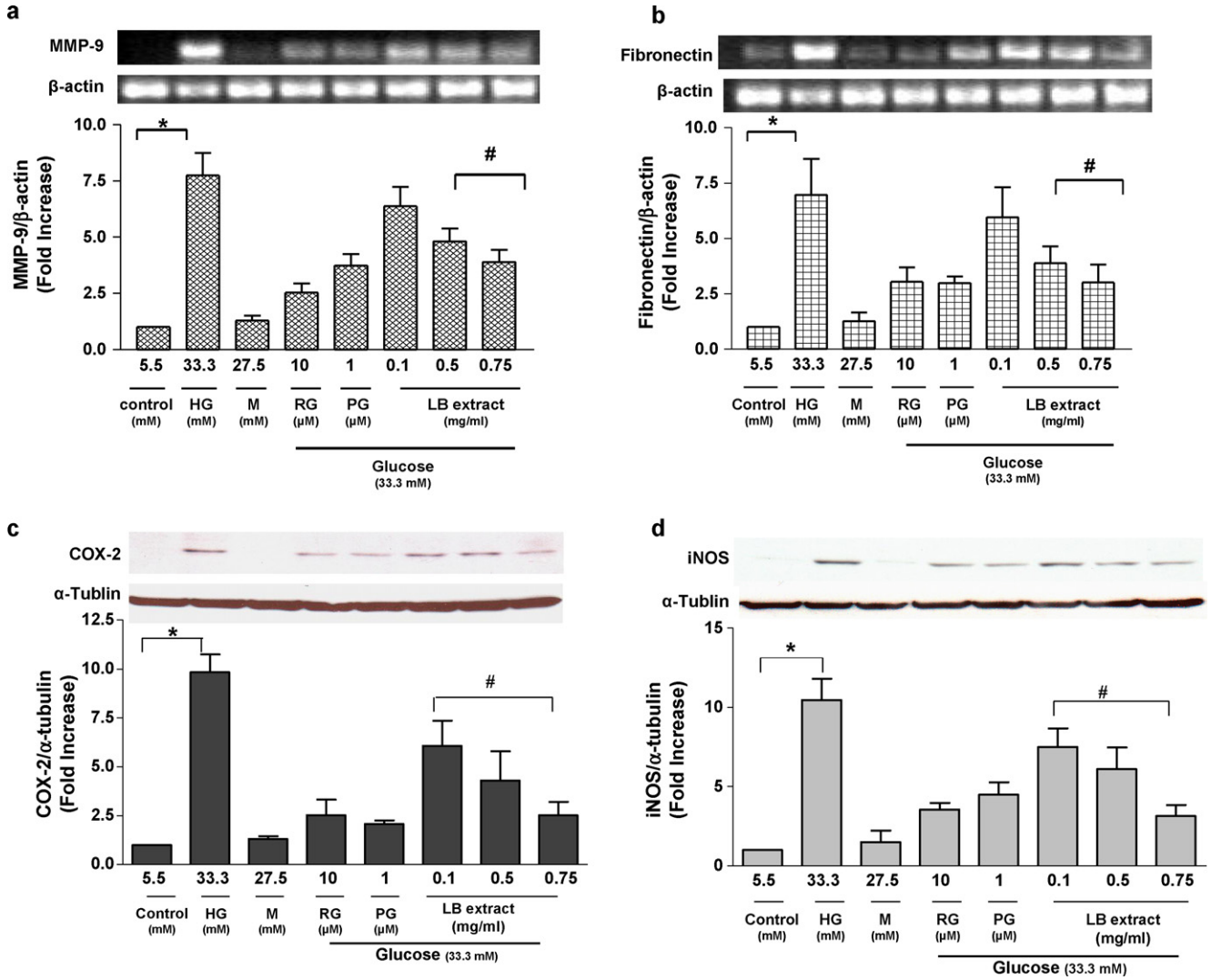

Fig. 3. Effects of LB extract on gene and protein expression of pro-inflammatory mediators in high glucose-treated ARPE-19 cells. The relative levels of mRNAs encoding for MMP9 (a) and fibronectin (b) were assessed by RT-PCR. mRNA bands were normalised to β-actin. The relative levels of protein encoding for COX-2 (c) and iNOS (d) were assessed by the immunoblotting method, and normalised to α-tublin. Levels in control were arbitrarily assigned a value of 1.0. Values are means ± SEM ($n = 4$). $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus high glucose (HG). Mannitol (M) served as osmotic control.

taurine concentration-dependently (1 μM–1 mM) increased PPAR-γ luciferase activity (Fig. 4a; 1.97-, 3.17-, 4.07- and 4.81-fold, respectively), and this was significantly suppressed by a selective PPAR-γ antagonist GW9226 (Fig. 4a). The RT-PCR analysis showed that taurine over this concentration range increased PPAR-γ mRNA expression dose-dependently, by 2.81-, 4.01- and 5.12-fold, respectively (Fig. 4c). Further, the results showed that taurine exhibited 2.75-, 4.41- and 5.55-fold increase in PPAR-γ protein expression by Western blotting analysis (Fig. 4d). The effects of taurine occurred in the concentration range that was found to be present in the active methanol fraction (i.e. 0.08–0.86 mM taurine in methanol fractions from 0.1 to 1 mg/ml).

3.5. Taurine down-regulates gene and protein expression of pro-inflammatory mediators in high glucose-treated ARPE-19 cells

To further understand the possible mechanism of taurine in improvement of DR through the PPAR-γ-induced anti-inflammatory pathway, we investigated the mRNA and protein expression of pro-inflammatory mediators in high glucose-induced ARPE-19 cells. Taurine (1 μM–1 mM) dose-dependently down-regulated the expression of pro-inflammatory mRNAs encoding MMP-9 (by 5.52-, 4.22- and 3.34-fold, respectively) (Fig. 5a) and fibronectin (by 5.86-, 4.38- and 3.65-fold, respectively) (Fig. 5b). Moreover, taurine dose-dependently decreased the protein expression of pro-inflammatory mediators encoding COX-2 (by 6.16-, 3.78- and 2.55-fold, respectively) (Fig. 5c) and iNOS (by 6.43-, 4.48- and 2.92-fold, respectively) (Fig. 5d).

3.6. Induced-fit docking

To understand the key interactions responsible for activity, the structure of taurine was flexibly docked into the ligand-binding site of PPAR-γ by the induced-fit docking method [54] using two crystal structures, 1FM9 and 1FM6, each of which has been induced by a different PPAR-γ agonist: farglitazar and rosiglitazone, respectively. Fig. 6a shows the plausible binding modes of taurine. Electrostatic interactions dominate with four hydrogen bonds between the negatively charged sulfonic acid and the polar residues of Y473, S289, H449, and H323. This network is similarly exhibited by farglitazar and rosiglitazone as seen in their respective crystal structures (Fig. 6b). The positively charged amine of taurine is predicted to hydrogen bond with Q286. The molecular volume occupied by taurine (410.8 Å^3) is significantly

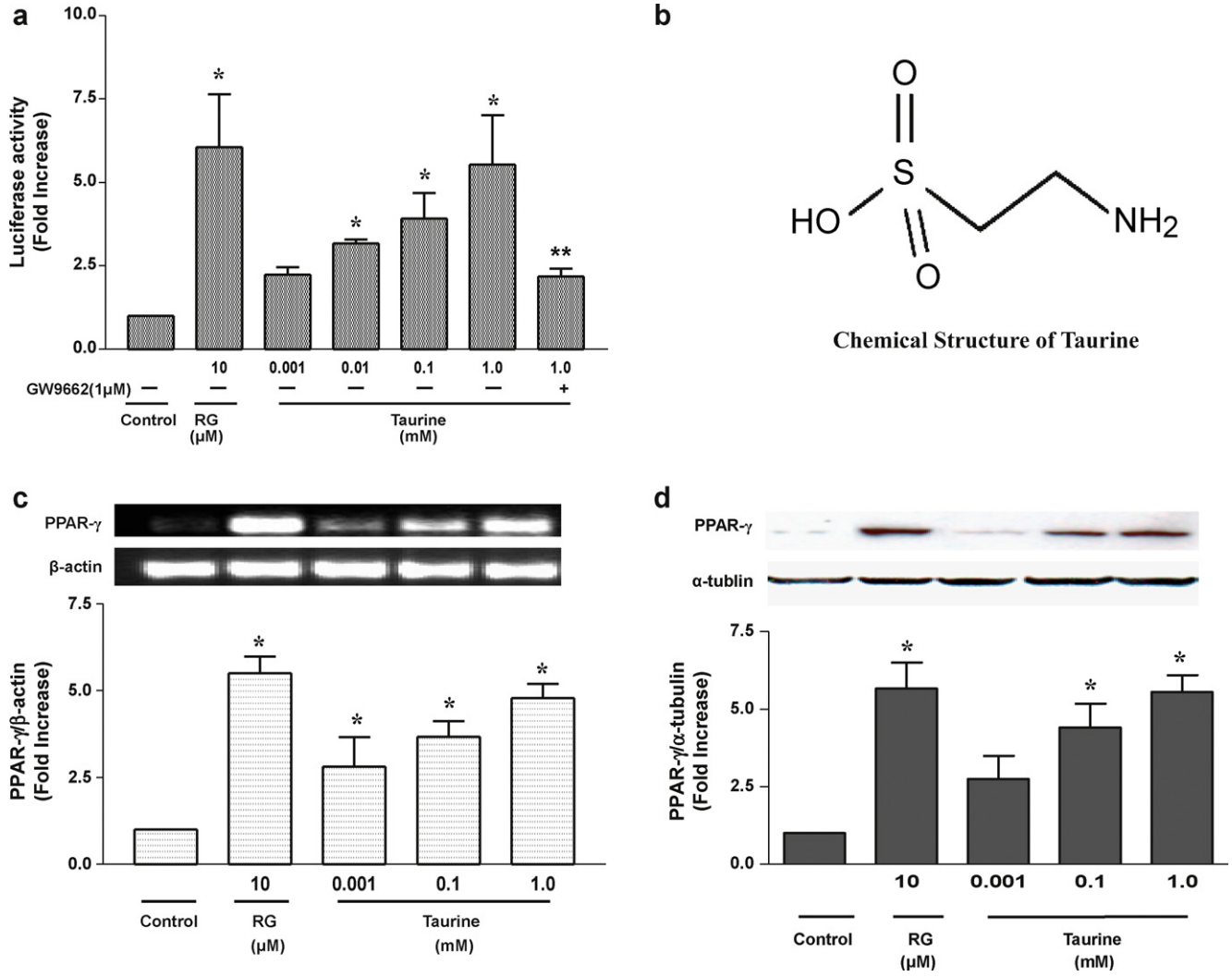

Fig. 4. Effects of taurine on PPAR-γ activity in cells in the *in vitro* models. (a) Taurine (0.001, 0.01. 0.1 and 1 mM) dose-dependently enhanced PPAR-γ luciferase activity transfected with reporter genes in HEK293 cells. Taurine-induced PPAR-γ luciferase activity is significantly suppressed by a selective PPAR-γ antagonist GW9226 (1 μM). Values are means ± SEM. (n = 4, each in triplicate). (c) mRNA and (d) protein expression of PPAR-γ in ARPE-19 cells treated with taurine (0.001, 0.1 and 1 mM) and RG (10 μM). The results were normalised to β-actin (for mRNA) or α-tublin (for protein). Control levels were arbitrarily assigned a value of 1.0. All values are means ± SEM (n = 4). $^*P < 0.05$ versus control, $^{**}P < 0.05$ versus taurine (1 mM) without GW9662.

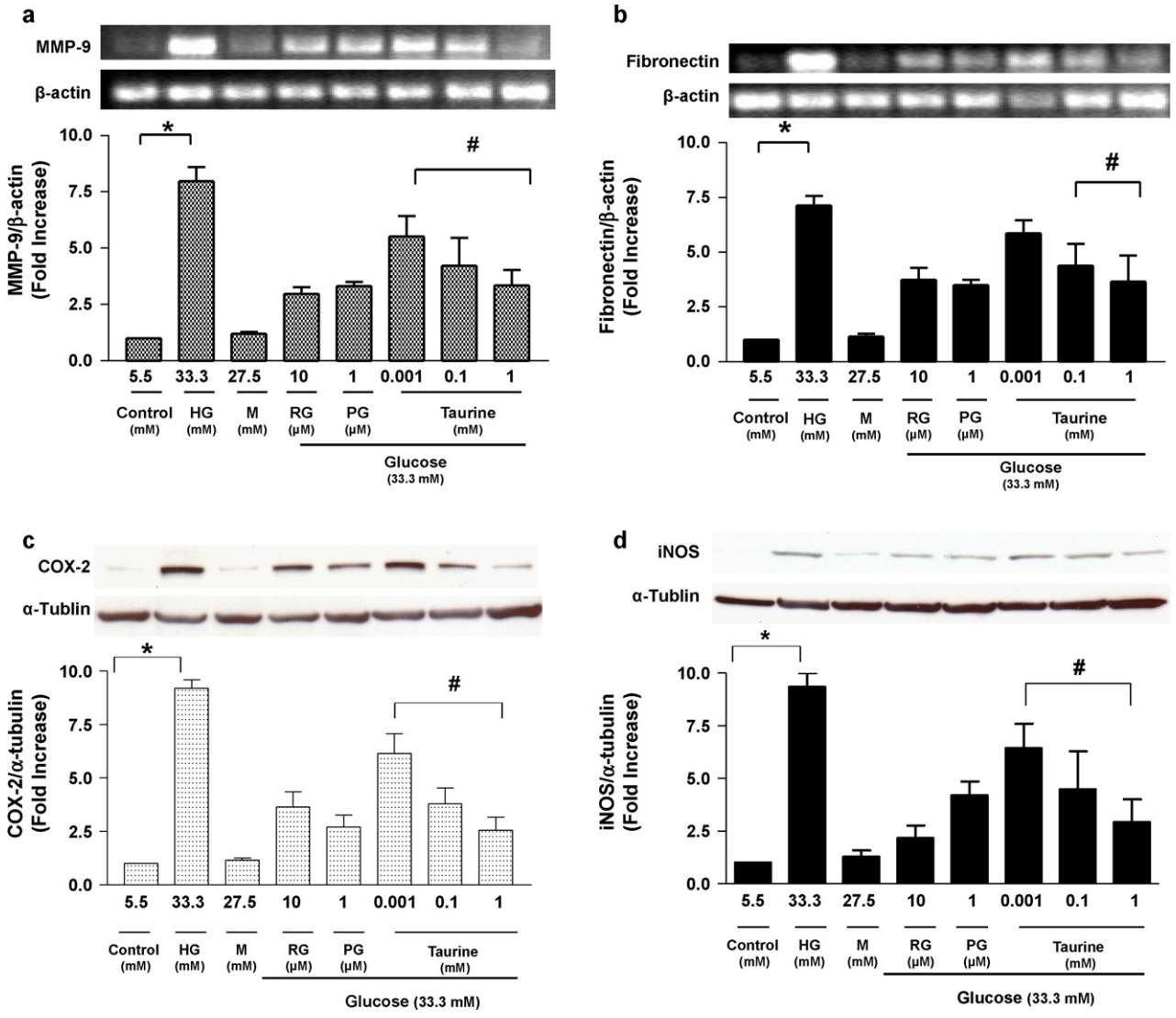

Fig. 5. Effects of taurine on gene and protein expression of pro-inflammatory mediators in high glucose-treated ARPE-19 cell. The relative levels of mRNAs encoding for MMP-9 (a) and fibronectin (b) were assessed by an RT-PCR method. Results were normalised to β-actin. The relative levels of protein encoding for COX-2 (c) and iNOS (d) were assessed by an immunoblotting method. Results were normalised to α-tublin. Levels in control were arbitrarily assigned a value of 1.0. All values are means ± SEM ($n = 4$). $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus high glucose (HG).

smaller than that of farglitazar (1712.3 Å^3) and rosiglitazone (1088.8 Å^3).

4. Discussion

Chronic hyperglycemia in type II diabetes is responsible for an array of microvascular complications that can lead to significant morbidity and mortality [59,60]. The prevalence of DR increases with the duration of diabetes, and more than 60% of those with type II diabetes have some degree of retinopathy after 20 years [61]. Therefore, early detection and prevention are desirable for the management of DR [62]. Laser photocoagulation therapy is the most common treatment modality for diabetic retinopathy. However, this therapy may damage neural tissue resulting in the deterioration of vision [63]. Therefore, development of new strategies for the management of excessive retinal vasopermeability and angiogenic changes are the basis for further research focus [64].

The underlying pathophysiological mechanisms associated with hyperglycemic-induced DR are through excessive formation of advanced glycation end products (AGEs) and production of excessive oxidative stress [16,65]. Moreover, these biochemical mechanisms lead to cascade events, such as promotion of inflammation and angiogenesis, which induce damage to diabetic retina, leading to DR [16,65]. Diabetes has been shown to be associated with up-regulation of various pro-inflammatory mediators in the retina, including MMP-9, fibronectin, COX-2 and iNOS [24,66].

PPAR-γ is heterogeneously expressed in the mammalian eye, and is most prominent in the retinal pigmented epithelium, photoreceptor outer segments and choriocapillaries [7–9]. A number of studies have shown that RPE might be the prime target for oxidative stress and PPAR-γ ligands modulate cellular defense against the oxidative stress [67]. Moreover, PPAR-γ ligands have shown therapeutic effects as modulators of inflammation by inhibiting the formations of AGEs [58,68,69]. In previous studies, PPAR-γ ligands were found to be potent inhibitors of corneal angiogenesis and neovascularization [11,12]. Consequently, RG was shown to inhibit both retinal leukostasis and retinal leakage by down-regulation of the adhesion molecule ICAM-1 [11]. Similarly, choroidal neovascularization was markedly reduced by intravitreous injection of troglitazone [9]. Moreover, recent studies

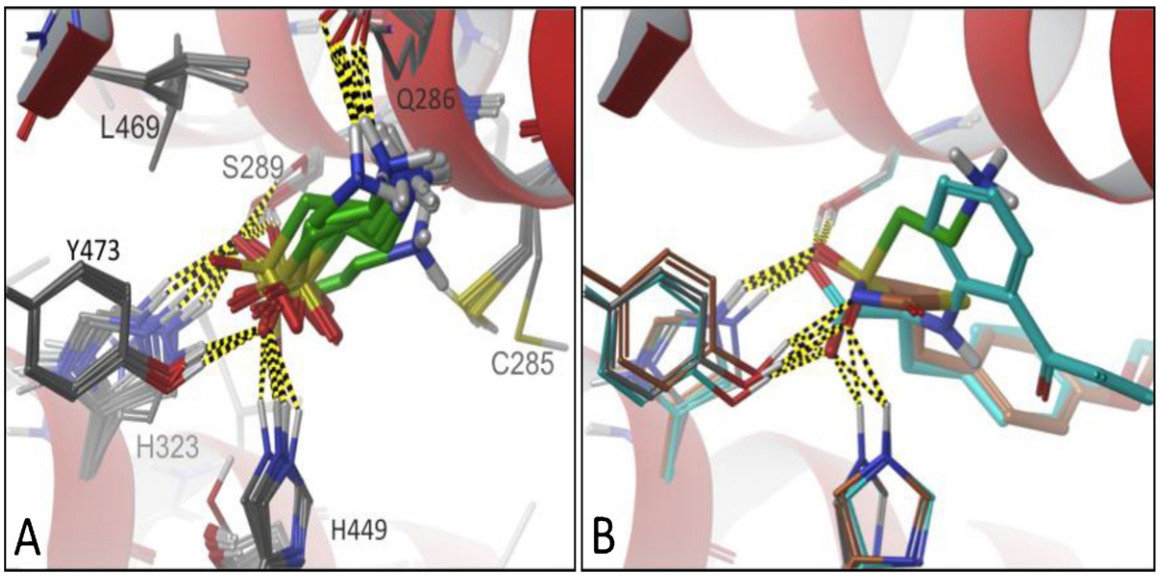

Fig. 6. View of PPAR-γ with predicted binding modes of taurine. (A) Best 10 scoring poses of taurine (green carbons) from induced-fit docking. (B) First ranked IFD pose of taurine (green carbons), superimposed with the crystal structures of 1FM6/rosiglitazone (orange carbons) and 1FM9/farglitazar (cyan carbons). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

have indicated that PG helps retinal pigment epithelial cells to maintain mitochondrial integrity by prevention of cytochrome c release and subsequent activation of the apoptosis pathway [70,71]. Observations of neonatal mice with retinal neovascularization have revealed that intravitreous injection of RG or troglitazone inhibited development of new retinal vessels [72]. Furthermore, a clinical trial has shown that RG delays the onset of proliferative diabetic retinopathy, possibly because of its anti-angiogenic activity [13].

In the traditional Chinese medicine literature, LB has been known for nourishing the eyes, livers and kidneys [73]. Previous human supplementation trials have indicated that the LB intake increases the fasting plasma zeaxathin levels, considered beneficial for maintaining macular pigment density in age-related macular degeneration [30]. LB polysaccharides (LBP) induce an activation of microglia (brain macrophages) in retina and have also been shown to be effective in glaucoma and modulating the immune system in retinal ganglion cells in a rat hypertension model [31,32]. Also, LBP have been shown to antagonize glutamate excitotoxicity in rat cortical neurons [74]. Taurine is a non-essential free amino acid, which is one of the chemical components abundantly present in LB and crosses the blood–retinal barrier [33,75]. A dietary source of taurine is essential for those animals (e.g. cat and humans) which cannot synthesize sufficient taurine and where greater consumption of taurine is required, such as in diabetes [76]. The concentration of taurine in photoreceptors, RPE cells and retina is estimated around 60–80 mM, which corresponds to about 40–75% of the total free amino acid content considered necessary to maintain physiological functions, including membrane stabilization, neuromodulation and integrity of retina [77–79]. Furthermore, several studies have reported that taurine potentiates the effect of insulin [35,36] and possibly affects the insulin receptor [37,38]. In addition, one study has indicated that high concentrations (20 mM) of taurine are capable of enhancing the phosphatidylinositide 3 (PI3)-kinase/Akt signaling pathway responsible for insulin-mediated stimulation of glucose transporter (GLUT4) activity and glucose uptake [80].

In the present study we hypothesised that LB extract and its taurine component may have PPAR-γ activity responsible for their effects in DR. In order to confirm our hypothesis, we investigated the effect of LB extract on PPAR-γ activity responsible for its effects in DR. Through bioassay and TLC analytical screening methods, we demonstrated that taurine present in LB extract appears to be the active component responsible for the PPAR-γ activation. Our results demonstrated for the first time that LB extract and its taurine component dose-dependently enhanced PPAR-γ luciferase activity in HEK293 cell line transfected with PPAR-γ reporter gene. This activity was significantly decreased by a selective PPAR-γ antagonist GW9662. Moreover, LB extract and its taurine component dose-dependently enhanced the expression of PPAR-γ mRNA and protein. In an inflammation model where ARPE-19 cells were exposed to high glucose LB extract and taurine down-regulated the mRNA expression of pro-inflammatory mediators encoding MMP-9, fibronectin, and the expression of COX-2 and iNOS proteins.

To delineate the key chemical interactions responsible for PPAR-γ activity, the molecular structure of taurine was simulated in the binding site of the PPAR-γ crystal structure via computational means. An induced-fit docking approach was used as it accounts for side-chain rearrangements induced by ligand binding. Whilst further work would be required to elucidate a truly accurate conformation of the taurine-PPAR-γ complex, which would require exhaustive computational approaches (e.g. molecular dynamics) or other experimental techniques (e.g. X-ray crystallography), the ensemble of 10 IFD poses presented here serves as an efficient guide into the plausible binding modes of taurine. The results suggest taurine's sulfonic acid serves as a bioisostere for the carboxylic acid and thiazolidinedione groups present in known PPAR-γ agonists like farglitazar and rosiglitazone. Because it similarly engages in electrostatic interactions with residues in the vicinity of the transcriptional activation function 2 domain, which is crucial for PPAR-γ activation [57], it is highly plausible that taurine activates PPAR-γ directly, supporting the experimental results. Taurine's overall weaker binding affinity is presumably a consequence of stronger desolvation penalty caused by its zwitterionic state, smaller size and the lack of hydrophobic functional groups seen in more potent agonists.

Overall, our results suggest that LB extract and its taurine component may be involved at least in part in delaying the progression of DR through activation of PPAR-γ. However, future effort will focus on research to further demonstrate the effectiveness of LB extract and its taurine component in the prevention of DR. Although synthetic PPAR-γ agonists, including rosiglitazone

have beneficial effects on diabetes, a trail of evidence indicates that long term intake of these agents may increase the risk of cardiovascular-related disease, such as myocardial infarction [81–84]. This issue has prompted the search for safe and novel compounds (of non-thiazolidione-related types) to manage diabetic complications, with herbal and natural products being an important source [85,86]. Taurine is endogenously produced by the human body, and often exogenously supplemented when there is deficiency to maintain the structural and functional integrity of retina [73,87–89]. Moreover, several studies have suggested that taurine has strong cardiovascular protective effects [90,91]. Therefore, more conclusive studies on the mechanism of action of taurine and its relation to its long term safety and efficacy in DR are warranted.

In summary, this study has shown that the traditional Chinese medicine *L. barbarum* and its taurine component enhance PPAR-γ activity in retinal cells, providing a rationale for the use of this valuable medicinal herb for the prevention of DR.

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