Effect of Tanshinone IIA in an In Vitro Model of Graves' Orbitopathy

Soolienah Rhiu,[1,2] Min Kyung Chae,[1] Eun Jig Lee,[3,4] Jong Bok Lee,[1] and Jin Sook Yoon[1]

[1]Department of Ophthalmology, Institute of Vision Research, Yonsei University College of Medicine, Seoul, Korea
[2]Department of Ophthalmology, Hallym University College of Medicine, Dongtan Sacred Heart Hospital, Hwaseong, Korea
[3]Department of Endocrinology, Brain Korea 21 Project for Medical Science, Institute of Endocrine Research, Severance Integrative Research Institute for Cerebral and Cardiovascular Disease, Seoul, Korea
[4]Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, Korea

Correspondence: Jin Sook Yoon, Department of Ophthalmology, Institute of Vision Research, Yonsei University College of Medicine, 50 Yonsei-Ro, Seodaemun-Gu, Seoul 120-752, Korea; yoonjs@yuhs.ac.

SR and MKC contributed equally to the work presented here and therefore should be regarded as equivalent authors.

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Purpose. We investigated the therapeutic effect of nontoxic concentrations of tanshinone IIA (TanIIA) from *Salvia miltiorrhiza* in primary cultures of orbital fibroblasts from Graves' orbitopathy (GO).

Methods. The effect of TanIIA on IL-1β–induced proinflammatory cytokine (IL-6, IL-8, MCP-1) expression was determined by real-time PCR. Antioxidant activity was investigated by measuring intracellular reactive oxygen species (ROS) generation stimulated by cigarette smoke extract (CSE) and heme oxygenase-1 (HO-1) expression. To evaluate antiadipogenic activity, fibroblasts were subjected to a differentiation protocol, including peroxisome proliferator activator gamma (PPARγ) agonist, for 10 days, and exposed to TanIIA during adipocyte differentiation. Differentiated cells were stained with Oil Red O, and the expression of adipogenesis-related factors, PPARγ, and CCAAT-enhancer-binding proteins (C/EBP) α and β were determined by Western blot.

Results. Expression of IL-6, IL-8, and MCP-1 mRNA was inhibited by TanIIA pretreatment in a dose-dependent manner in GO orbital fibroblasts ($P < 0.05$). Tanshinone IIA decreased CSE- or H_2O_2-induced ROS levels in a dose-dependent manner and upregulated HO-1 protein expression in a time- and dose-dependent manner ($P < 0.001$). Treatment of orbital fibroblasts with TanIIA increased phosphorylated extracellular signal-regulated kinase (pERK), and an ERK inhibitor significantly blocked TanIIA-induced HO-1 upregulation. Adipogenesis was inhibited by TanIIA in a dose-dependent manner ($P < 0.001$), as evidenced by Oil Red O stain and decreased PPARγ and C/EBPα expression in Western blot analysis.

Conclusions. Our study results suggest that TanIIA possesses significant anti-inflammatory, antioxidant, and antiadipogenic effects in primary orbital fibroblasts. These results provide the basis for further study of the potential use of TanIIA to treat GO. Tanshinone IIA showed significant anti-inflammatory, antioxidant, and antiadipogenic effects in primary orbital fibroblasts from Graves' orbitopathy patients. These results provide the basis for further study of the potential use of tanshinone IIA to treat Graves' orbitopathy.

Key words: adipogenesis, Graves' orbitopathy, heme oxygenase-1, inflammation, orbital fibroblasts, oxidative stress, tanshinone IIA

Graves' disease is an autoimmune disease of the thyroid gland in which autoantibodies bind to the thyrotropin receptor on thyroid follicular cells, thereby activating gland function and leading to excess production of thyroid hormones.[1] Up to 50% of patients with Graves' disease develop pathological manifestations in the eye, known as Graves' orbitopathy (GO).[2] Enlargement of extraocular muscle bodies together with an increase in orbital connective/fatty tissue within the bony orbits is responsible for most of the orbital complications in patients with moderate to severe GO.[3] In addition, an overabundance of adipose tissue within the orbits is another prominent feature of GO. It is likely that orbital adipose tissue in GO is more cellular and comprises a higher proportion of preadipocytes capable of differentiating into adipocytes.[4] The process of adipocyte differentiation appears to be a phenotypic attribute of orbital fibroblasts that is not observed in dermal and perimysial fibroblasts. The mechanistic connections between the pathogenic components in GO are poorly understood.

Oxidative stress has also been implicated in the pathogenesis of GO, and cigarette smoking is known to be one of the major environmental factors affecting GO.[5] Cigarette smoke is considered to act, in part, by enhancing the generation of reactive oxygen species (ROS) and increasing oxidative stress in the closed bony orbital environment, either through direct contact with the sinus and medial wall, or indirectly through the bloodstream.[5] However, the contribution of ROS to the pathogenesis of GO is unclear.

There is no completely reliable, specific, and safe medical therapeutic agents for moderate to severe GO. Glucocorticoids

are indicated as the first-line treatment due to their anti-inflammatory and immunosuppressive actions, alone or in combination with orbital radiotherapy.[6] However, the management of moderate to severe GO is challenging, and often not satisfactory. Previous treatments, such as high-dose glucocorticoids and/or orbital radiotherapy, are mostly effective in patients with severe and active eye disease.[7] Soft tissue inflammatory changes, recent-onset extraocular muscle involvement, and optic neuropathy are the most responsive to glucocorticosteroids, whereas proptosis and long-standing extraocular muscle involvement associated with fibrotic changes are poorly influenced. Adverse effects and complications caused by systemic glucocorticoid therapy also should not be ignored.

Tanshinone, isolated from *Salvia miltiorrhiza bunge* (danshen), has been widely used to treat coronary, cerebrovascular, and cardiovascular disease in traditional Chinese medicine. Owing to its purported biological activity and lack of serious side effects, as confirmed by pharmacological investigations and clinical use, a number of traditional Chinese medicinal preparations have been developed containing danshen.[8] Tanshinone IIA (TanIIA), a lipophilic diterpene, is the most abundant active ingredient and is structurally representative of tanshinone.[9] It is available as a purified sulfonate salt for use in stroke, heart attack, and angina patients,[10,11] and can reduce the brain infarct volume in transient focal cerebral ischemia in mice. It is suggested that the therapeutic effect of TanIIA on atherosclerosis may result from its antioxidant and anti-inflammatory properties.[12,13] Tanshinone IIA is also reported to have antifibrotic[14] and antiadipogenetic[15,16] effects.

Tanshinone IIA has recently been found to inhibit 3T3-L1 preadipocyte differentiation and transcriptional activity in full-length peroxisome proliferator activator gamma (PPARγ) and PPARγ ligand-binding domains.[15] Based on reports that TanIIA showed anti-inflammatory, antioxidant, and antiadipogenesis effects, we considered that TanIIA may have inhibitory effects on the main pathogenic pathways of GO.

In this study, we investigated the effect of TanIIA on the expression of proinflammatory genes and on adipocyte differentiation, and its antioxidant effects, in primary cultures of orbital fibroblasts from patients with GO.

Patients and Methods

Reagents

The regents were purchased as follows: TanIIA (Korea Food & Drug Administration, Osong Health Technology Administration Complex, Cheongju, Korea); Dulbecco's modified Eagle's medium (DMEM):F12 (1:1), fetal bovine serum (FBS), penicillin, and gentamycin (Hyclone Laboratories, Inc., Logan, UT, USA); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Oil Red O, and 2′-animo-3′-methoxyflavone (PD98059) (Sigma-Aldrich Corp., St. Louis, MO, USA); bromodeoxyuridine (5-bromo-2-deoxyuridine [BrdU]) cell proliferation assay kit (Chemicon, Temecula, CA, USA); annexin V–FITC apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA); recombinant human (rh) IL-1β (R&D Systems, Minneapolis, MN, USA); anti-heme oxygenase (HO)-1, anti-phospho-extracellular-regulated kinase (ERK) 1/2, anti-ERK 1/2, anti-phospho-Akt, anti-phospho-phosphatidylinositol 3-kinase (PI3-K), and anti-phospho-c-Jun N-terminal kinase (JNK) (Cell Signaling, Danvers, MA, USA); and anti-PPARγ antibody, anti-CCAAT-enhancer-binding protein (C/EBP) α antibody, anti-C/EBP β antibody, anti-HO–1 antibody, and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell and Tissue Culture Protocols

Orbital adipose/connective tissue specimens were obtained in the course of orbital decompression surgery for severe GO ($n = 6$; four women and two men, aged 25–58 years). Normal orbital adipose/connective tissue specimens were collected in the course of orbital surgery for other noninflammatory problems from patients with no history of thyroid disease or GO and with no clinical evidence of GO ($n = 4$; three women and one man, aged 31–67 years). The GO patients had not received steroid medication for at least 3 months before surgery, and were euthyroid at the time of surgery. The clinical activity scores at the time of tissue harvest were below 4 in all patients. None of the patients had been treated previously with orbital radiotherapy. The research followed the tenets of the Declaration of Helsinki and was approved by the institutional review board of Severance Hospital (4-2011-05-90), Yonsei University College of Medicine, Seoul, Korea, and all study participants gave their written informed consent.

Orbital fibroblast cultures were established in accordance with published methods.[17] Tissue explants were minced and placed directly in plastic culture dishes to allow preadipocyte fibroblasts to proliferate. Cells were incubated in DMEM:F12 (1:1) containing 20% FBS, penicillin (100 U/mL), and gentamycin (20 μg/mL) in a humidified 5% CO2 incubator at 37°C, and maintained in two 80-mm flasks with DMEM:F12 (1:1) containing 10% FBS and antibiotics. Monolayers were passaged serially by gently treating with trypsin/EDTA. The strains were stored in liquid N2 until needed and used between the first and fifth passages. Cultured orbital fibroblasts were pretreated with TanIIA before incubation with rh IL-1β to study the suppressive effect of TanIIA on inflammation.

Cell Viability and Apoptosis Assays

To evaluate the effect of TanIIA on cell viability, orbital fibroblasts were seeded into 24-well culture plates (1×10^5 cells/well) and treated with different concentrations of TanIIA (0–30 μM) for 6 and 24 hours. After treatment, cells were washed, incubated with 5 mg/mL MTT solution for 3 hours at 37°C, then solubilized in ice-cold isopropanol and analyzed spectrophotometrically. The absorbance of the dye was measured at 560 nm, with background subtraction at 630 nm, with a microplate reader (EL 340 Biokinetics Reader; Bio-Tek Instruments, Winooski, VT, USA).

To evaluate the effect of TanIIA on the apoptosis of orbital fibroblasts, an annexin V–FITC kit was used to detect apoptotic cells. Cells were washed with PBS and incubated in serum-free DMEM in the presence of TanIIA at 0 to 30 μM for 6 and 24 hours. Cells were washed and incubated for 15 minutes at room temperature in the presence of annexin V labeled with FITC and propidium iodide (PI). In total, 10,000 cells were excited at 488 nm, and emission was measured at 530 and 584 nm to assess FITC and PI fluorescence, respectively.

Lactate Dehydrogenase (LDH) Assay

The cytotoxicity of TanIIA in tissue culture was determined using the LDH leakage assay. To prepare for the LDH assay, orbital fat tissues were cultured with media containing varying TanIIA concentrations (0–30 μM) for 6, 24, or 48 hours. The medium was transferred to a 1.5-mL microcentrifuge tube and centrifuged at 12,000*g* and 4°C for 15 minutes to remove cell debris. Then, 100 μL of each sample was added to the substrate solution and the absorbance at 490 nm was measured using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). Activity of LDH was obtained by measuring the

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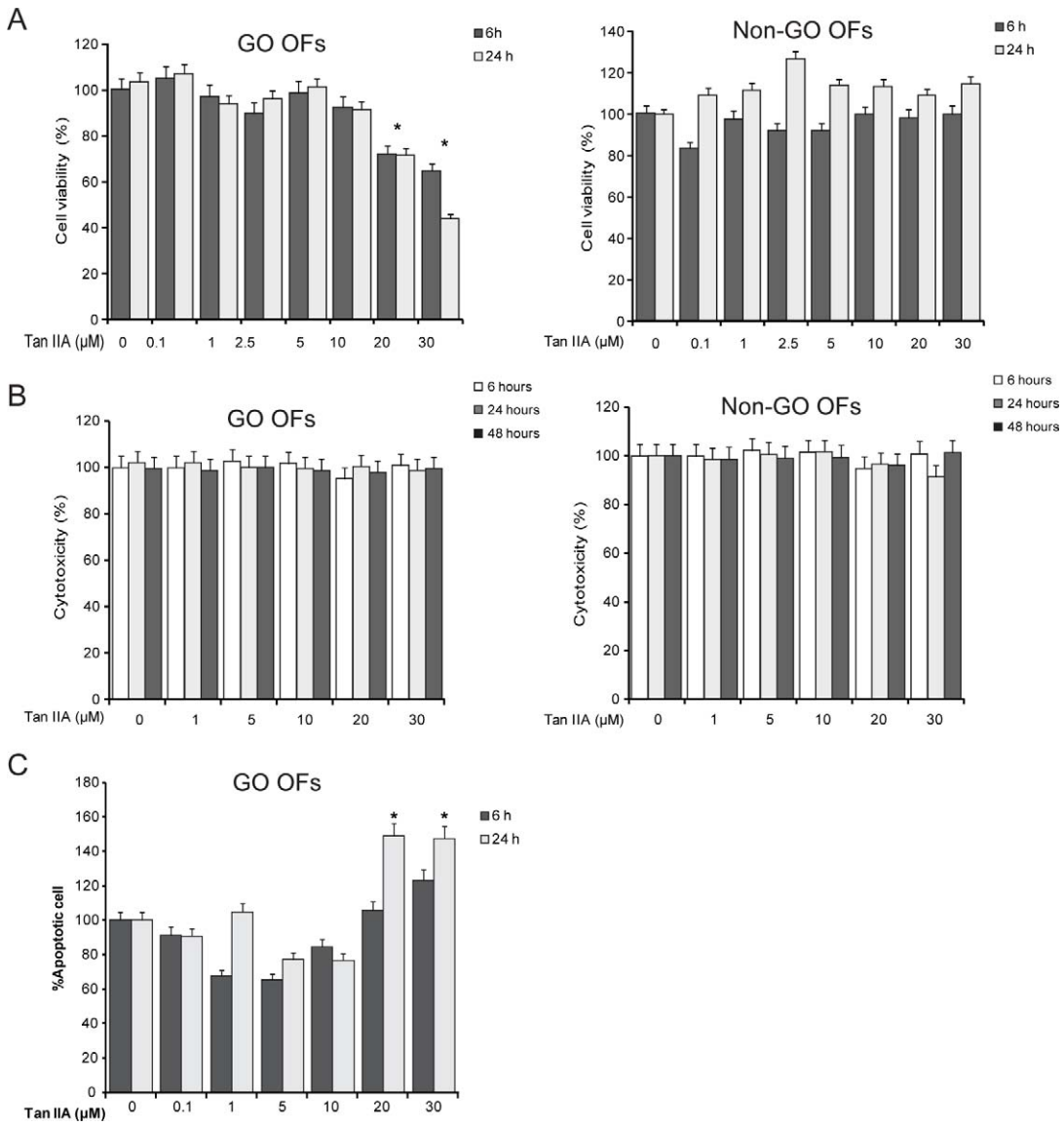

Figure 1. Effect of TanIIA on the viability of orbital fibroblasts. (**A**) Orbital fibroblasts (1×10^5) of GO patients and normal control (non-GO) individuals were seeded in 24-well culture plates and treated with different concentrations of TanIIA (0–30 μM) for 6 and 24 hours. After treatment, assays with MTT were performed to test for viability. (**B**) Orbital fibroblasts from GO patients and non-GO individuals were cultured in conditioned media containing increasing doses (0–30 μM) of TanIIA for different treatment times (6, 24, and 48 hours). After treatment, toxicity was determined by measuring LDH release. (**C**) Orbital fibroblasts of GO patients were incubated with TanIIA at 0 to 30 μM for 6 and 24 hours. An annexin V–FITC kit was used to detect phosphatidylserine externalization, as an index of apoptosis. Percentage of stained cells with annexin V was analyzed by flow cytometry. Results are expressed as percentage of untreated control values presented as mean ± SD. Assays were performed in triplicate with cells from three different donors. Primary orbital cells at passages 1 to 5 were used. Differences between treated and untreated cells are indicated (in contrast to cells not treated with TanIIA, $^*P < 0.05$).

decrease in nicotinamide adenine dinucleotide absorbance over time (slopes).

Quantitative Real-Time PCR

Total RNA (1 μg) was isolated and reverse transcribed into complementary DNA according to the manufacturer's instructions (#74104; Qiagen, Valencia, CA, USA). The resulting cDNA was amplified on an ABI 7300 real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA) using the TaqMan universal PCR master mix and recommended PCR conditions to quantitatively assess gene transcript levels in the cell samples. All PCR reactions were performed in triplicate. The catalog numbers (#639543; Clontech Laboratories–Takara Bio, Mountain View, CA, USA) of the primers were Hs00985639_m1 for IL-6, Hs00174103_m1 for IL-8, Hs00234140_m1 for monocyte chemotactic protein (MCP)-1, and H299999905_ml for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH expression was used for normalization, and the results are expressed as fold change in the threshold cycle (Ct) value relative to the control group using the $2^{-\Delta\Delta Ct}$ method.[18]

Intracellular ROS Measurement

Cigarette smoke extract (CSE) was prepared in accordance with published methods,[5] using commercially available, filtered cigarettes (Marlboro 20 class A cigarettes, made in Korea by Philip Morris Korea, Inc. (Seoul, Korea), and

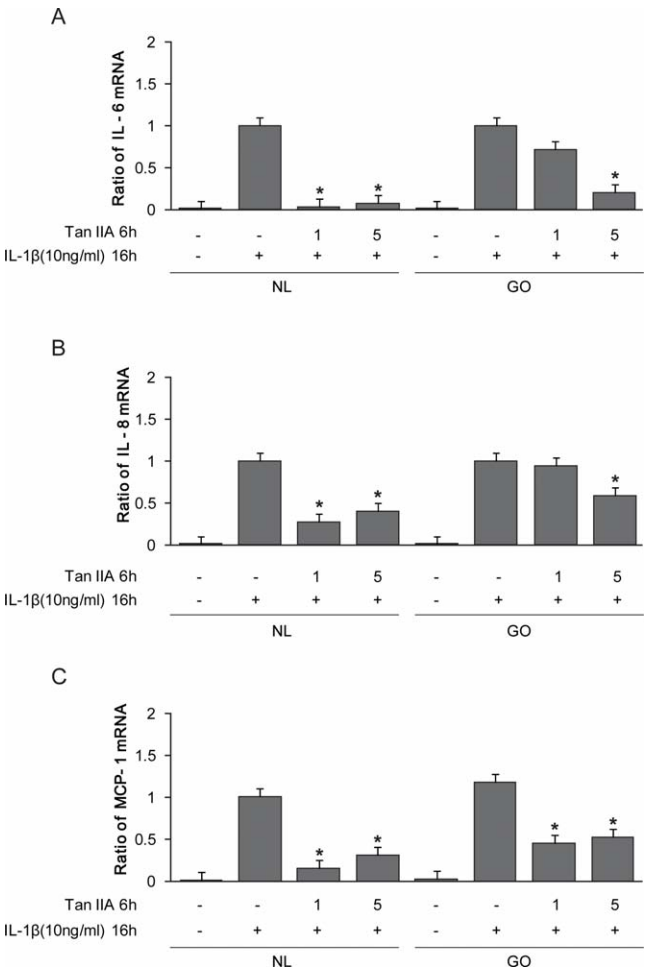

Figure 2. Effect of TanIIA on tissue mRNA transcript levels of IL-6, IL-8, and MCP-1. After the treatment of tissue cultures with various concentrations of TanIIA for 6 hours, total RNA (1 μg) was isolated and reverse transcribed into cDNA, which was amplified for IL-6, IL-8, and MCP-1 mRNA by real-time PCR. Gene transcript levels of IL-6 (**A**), IL-8 (**B**), and MCP-1 (**C**) are shown as mean ± SD fold depression in cytokine mRNA levels relative to control samples without TanIIA treatment. Experiments were performed in triplicate with cells from three different donors. Primary orbital cells at passages 1 to 5 were used. Differences between treated and untreated cells are indicated (in contrast to cells not treated with TanIIA, $^*P < 0.05$). NL, normal control.

containing 8.0 mg of tar and 0.7 mg of nicotine). The noncytotoxic doses of CSE and H_2O_2 were determined as previously described,[5] using the MTT assay. A dose of 2% CSE or 200 μM H_2O_2 was determined to be noncytotoxic.

Release of ROS was determined with 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Inc., Eugene, OR, USA), an oxidant-sensitive fluorescent probe, as previously described.[19] The H_2DCFDA is deacetylated intracellularly by esterase, forming H_2DCF, which is oxidized by ROS to 2′,7′-dichlorofluorescein (DCF), a highly fluorescent compound. Orbital fibroblasts were seeded on glass-bottomed 60-mm^2 dishes overnight and pretreated with TanIIA (0–10 μM) for 24 hours in the presence or absence of 2% CSE or 200 μM H_2O_2 as described above for 1 hour. Cells were then incubated with 10 μM H_2DCFDA (Invitrogen, Eugene, OR, USA) in growth medium for 30 minutes at 37°C. Cells were washed with PBS, and analyzed with a flow cytometer (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA).

Adipogenesis

Orbital fibroblasts were exposed to a differentiation protocol according to our previous reports[5,17] to enhance adipogenesis. The cells were grown to confluence in six-well plates, and then exposed to differentiation medium for 10 days. The culture medium was changed to serum-free DMEM-glucose supplemented with 33 μM biotin, 17 μM pantothenic acid, 10 μg/mL transferrin, 0.2 nM T3, 1 μM insulin (Boehringer-Mannheim, Manheim, Germany), 0.2 μM carbaprostaglandin (cPGI2; Calbiochem, La Jolla, CA, USA), and a PPARγ agonist, rosiglitazone 10 μM (Cayman, Ann Arbor, MI, USA). For the first 4 days, 1 μM insulin, 10 μM dexamethasone, 0.1 mM isobutylmethylxanthine (IBMX) and differentiation media was used and after the first 4 days, dexamethasone and IBMX were excluded from the media. Rosiglitazone 10 μM was added from day 1 for further stimulation of adipogenesis. The differentiation protocol was continued for 10 days, during which the medium was replaced every 2 to 3 days. To evaluate the effect of TanIIA on adipocyte differentiation, we exposed cultures to TanIIA (0, 1, 5, 10 μM) for the entire 10-day differentiation period.

Oil Red O Staining

Cells were stained with Oil Red O as described by Green and Kehinde.[20] A 0.5% Oil Red O stock solution was prepared and passed through a 0.2-μm filter. To prepare the working solution, 6 mL stock solution was mixed with 4 mL distilled water, left for 1 hour at room temperature, and filtered through a 0.2-μm filter. Cells were washed twice with 1× PBS, fixed with 10% formalin in PBS for 1 hour at 4°C, and stained with 300 μL Oil Red O working solution for 1 hour at room temperature. The dishes were washed with distilled water before being visualized using an Axiovert (Carl Zeiss, Jena, Germany) light microscope and photographed at ×40 and ×100 using an Olympus BX60 light microscope (Olympus, Melville, NY, USA).

To measure lipid accumulation, cell-bound Oil Red O was solubilized with 100% isopropanol, and the optical density of the solution was measured with a spectrophotometer at 490 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors, and the results were normalized to the absorbance of untreated control differentiated cells.

Western Blot Assay

Differentiated cells were washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% [vol/vol] glycerol, 10 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% [vol/vol] Triton X-100; Sigma-Aldrich Corp.) on ice for 30 minutes. Lysates were centrifuged for 10 minutes at 12,000*g* and the cell homogenate fractions stored at −70°C before use.

Protein concentrations in the supernatant fractions were determined by the Bradford assay. Equal amounts of protein (50 μg) were boiled in sample buffer and resolved by 10% (wt/vol) SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Billerica, MA, USA), probed overnight with primary antibodies in Tris-buffered saline containing Tween 20 (TBST), and washed three times with TBST. Immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed to X-ray film (Amersham Pharmacia Biotech).

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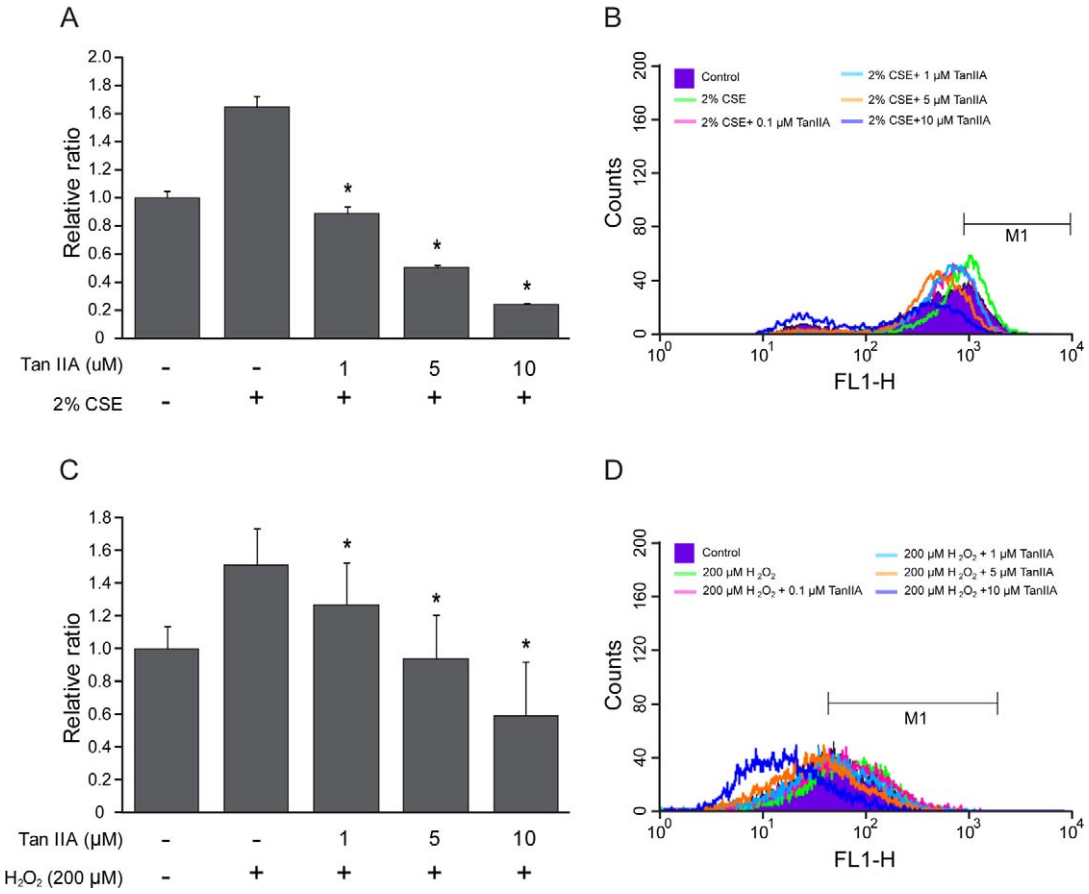

Figure 3. Tanshinone IIA decreases ROS levels in orbital fibroblasts from GO. Orbital fibroblasts were incubated with 10 μM H_2DCFDA for 30 minutes in various concentrations of TanIIA (0–10 μM) in the presence or absence of 2% CSE (**A**, **B**) or 200 μM H_2O_2 (**C**, **D**) for 1 hour, and the fluorescence intensities were analyzed using FACS. Results are expressed as percentage of untreated control values presented as mean ± SD (**A**, **C**). Flow cytometry plots are presented (**B**, **D**). Assays were performed in triplicate with cells from three different GO donors. Primary orbital cells at passages 1 to 5 were used. Differences between treated and untreated cells are indicated (in contrast to cells not treated with TanIIA, $^*P < 0.001$).

Statistical Analysis

All values are expressed as the mean ± SD of three separate experiments, using cells from three different individuals. Statistical analysis was performed with repeated measures ANOVA and independent *t*-test with Bonferroni test as a post hoc test to identify differences between control and drug-treated samples using the SPSS program for Windows, version 20.0 (SPSS, Inc., Chicago, IL, USA). A *P* value less than 0.05 was considered significant.

Results

Effect of TanIIA on the Viability of Orbital Fibroblasts

To determine the nontoxic concentrations of TanIIA in orbital fibroblasts, the MTT assay, LDH cytotoxicity assay, and annexin V-FITC apoptosis assay were performed. Exposure of cells to 10 μM or less of TanIIA for 6 and 24 hours did not decrease cell viability below 95% in both normal and GO orbital fibroblasts (Fig. 1A). Release of LDH from cells treated with TanIIA (0–30 μM) for 6, 24, and 48 hours did not decrease cell viability in both normal and GO orbital fibroblasts (Fig. 1B). Exposure of cells to 10 μM or less of TanIIA for both 6 and 24 hours did not induce a significant level of apoptosis or necrosis in GO orbital fibroblasts (Fig. 1C). Therefore, noncytotoxic concentrations (0–10 μM) of TanIIA were used to study the therapeutic effect of TanIIA in Graves' orbital fibroblasts in this study.

Effect of TanIIA on the Expression of mRNA of IL-1β-Induced Proinflammatory Cytokines

We investigated the effects of increasing treatment doses of TanIIA on IL-6, IL-8, and MCP-1 mRNA expression in response to IL-1β (10 μg/mL, 16 hours) challenge of orbital fibroblasts from GO and normal orbital fibroblasts. The 16-hour time point was chosen based on previous dose-response and time-course studies that indicated upregulation of IL-6, IL-8, and MCP-1,[17,21,22] and also confirmed by data from our experiments (Supplementary Fig. S1). Pretreatment with 5 μM TanIIA for 6 hours resulted in a significant inhibition of IL-6, IL-8, and MCP-1 mRNA expression induced by IL-1β in both normal and GO orbital fibroblasts ($P < 0.05$, Fig. 2).

TanIIA Treatment Reduced CSE- and H_2O_2-Stimulated ROS Production in GO Orbital Fibroblasts

Reactive oxygen species production detected by a ROS-sensitive fluorescent probe, DCFDA in GO orbital fibroblasts stimulated with either 2% CSE or 200 μM H_2O_2 for 1 hour was

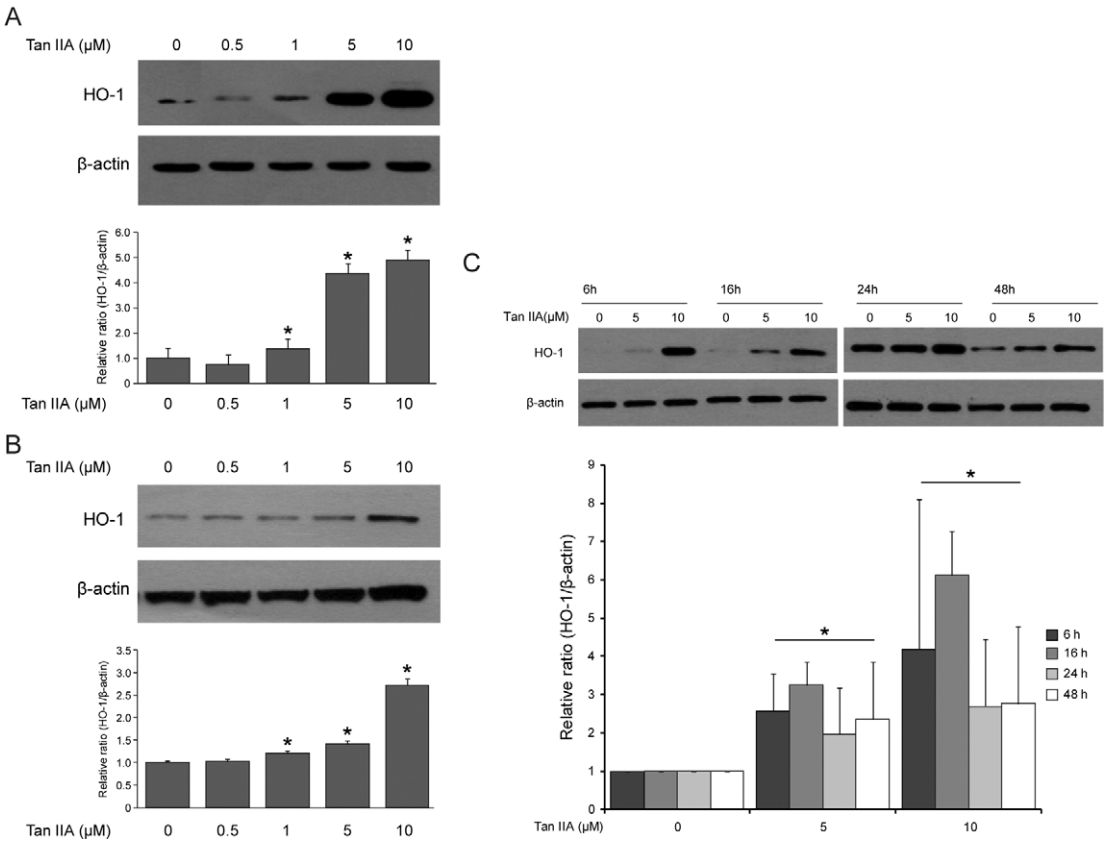

Figure 4. Dose-dependent effect of TanIIA in HO-1 expression in orbital fibroblasts. After incubating orbital fibroblasts with various concentrations of TanIIA (0–10 μM) for 24 hours and subsequently washing them twice with PBS, cell lysates were electrophoresed and probed by Western blot with HO-1– and β-actin–specific antibodies. Data were normalized to the β-actin level, and the relative densities were quantified with a densitometer. Results are representative of three separate experiments of GO (**A**) and normal control (**B**) patients (in contrast to cells not treated with TanIIA, $^*P < 0.001$). (**C**) After incubating orbital fibroblasts from GO with various concentrations of TanIIA (0–10 μM) and various time conditions (6–48 hours) and then washing twice with PBS, cell lysates were electrophoresed and probed by Western blot with HO-1– and β-actin–specific antibodies (in contrast to cells not treated with TanIIA, $^*P < 0.05$). Triplicate measurements from three different donors were averaged, and data are expressed as mean ± SD. Primary orbital cells at passages 1 to 5 were used.

significantly suppressed by TanIIA pretreatment in a dose-dependent manner (all $P < 0.001$; Fig. 3).

Effect of TanIIA on HO-1 Expression in Orbital Fibroblasts

Because the production of HO-1, an antioxidant enzyme, may lead to changes in intracellular redox status, we investigated whether TanIIA induces HO-1 expression in orbital fibroblasts. The expression of the HO-1 protein was significantly upregulated on treatment with TanIIA (1–10 μM) for 24 hours in a dose-dependent manner in both GO (Fig. 4A) and normal orbital fibroblasts (Fig. 4B). In GO orbital fibroblasts, the amount of HO-1 protein production by TanIIA was stronger than that in normal orbital fibroblasts ($P = 0.004$). Orbital fibroblasts were also treated with various concentrations of TanIIA (0–10 μM) and various time conditions (6–48 hours) and showed a dose-dependent effect on HO-1 protein expression in GO orbital fibroblasts (Fig. 4C).

Signaling Pathways of TanIIA-Induced HO-1 Expression in Orbital Fibroblasts

To investigate the molecular mechanism of HO-1 induction by TanIIA in orbital fibroblasts, we observed the effect of TanIIA on ERK1/2, Akt, PI3-K, and JNK activation. The data in Figures 5A and 5B show that 10 μM TanIIA provoked a rapid and relevant activation of ERK1/2, whose phosphorylation was maximal at 30 minutes in GO cells and at 15 minutes in non-GO cells, and declined to basal levels at 180 minutes; PI3-K phosphorylation was suppressed by TanIIA treatment in both GO and non-GO cells; JNK phosphorylation was enhanced only in non-GO cells with a time-course similar to that observed for EKR1/2; the phosphorylation of Akt was enhanced at the 5-minute challenge in GO cells.

Thus, ERK1/2 was identified as a target of TanIIA, with its phosphorylation being strongly enhanced at 15- to 30-minute challenge. To elucidate the upstream signaling events leading to the induction of HO-1 expression in TanIIA-treated orbital fibroblasts, different kinase inhibitors were used, including PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), LY294002 (PI3-K inhibitor), and U0126 (MAPK inhibitor). The PD98059 significantly blocked phosphorylation of ERK 1/2 in GO orbital fibroblasts pretreated with 10 μM TanIIA for 16 hours ($P < 0.001$; Fig. 5C). When cells were pretreated with PD98059, TanIIA-induced HO-1 was also significantly inhibited ($P < 0.001$; Fig. 5D), suggesting that activation of the ERK signaling pathway was required for the TanIIA induction of HO-1 protein expression in orbital fibroblasts.

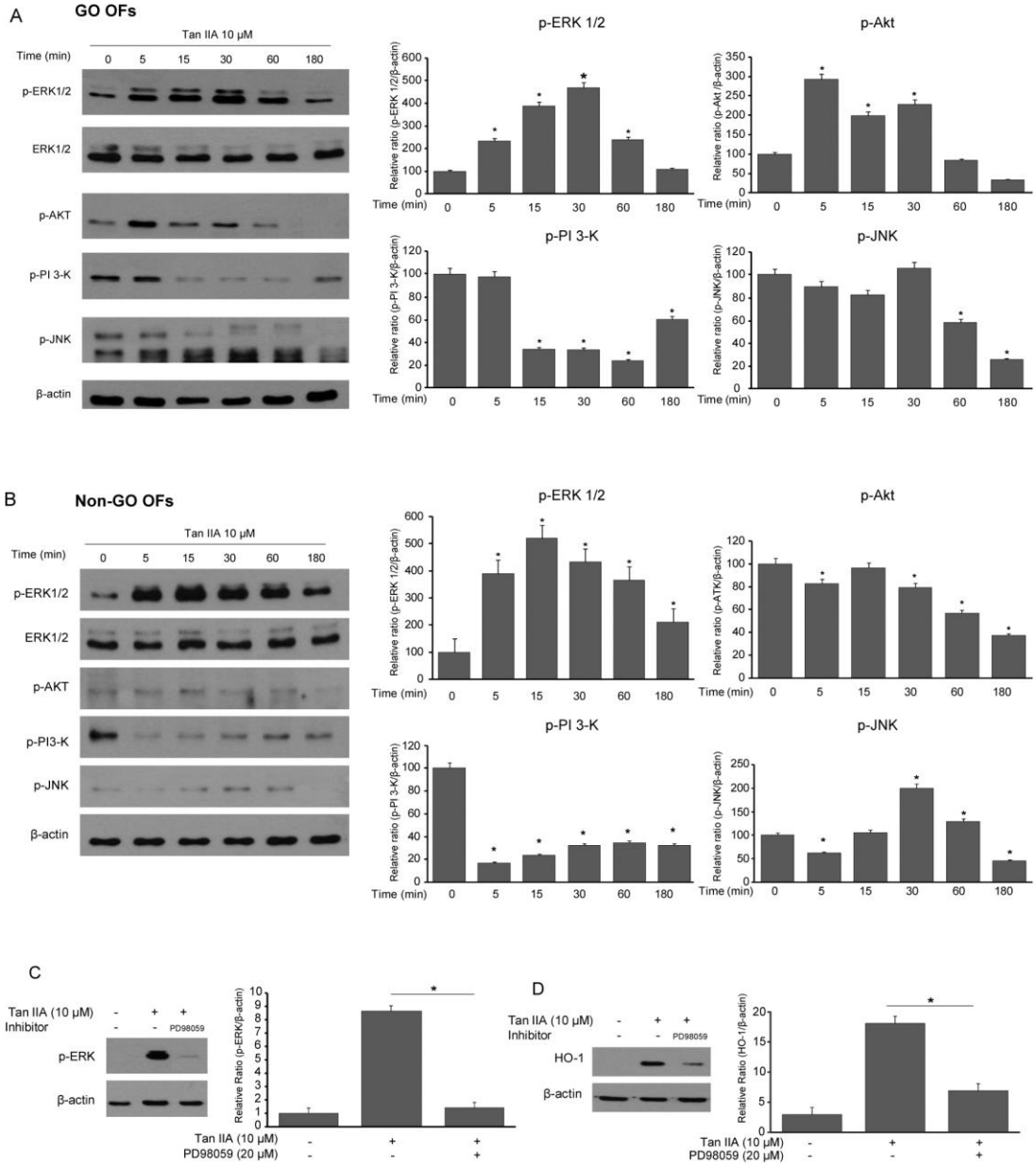

Figure 5. Signaling pathways of TanIIA-induced HO-1 expression in orbital fibroblasts. (**A**) Effects of TanIIA on ERK 1/2, Akt, PI3-K, and JNK phosphorylation in orbital fibroblasts of GO. Cells were treated with TanIIA (10 μM) for various time periods and lysed. Cell lysates were electrophoresed and probed by Western blot with phospho-ERK 1/2, ERK 1/2, phospho-Akt, phospho-PI3-K, phospho-JNK, and β-actin-specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin. Quantification by densitometry, normalized to the β-actin level in the same sample, is shown for phospho-ERK 1/2, phospho-Akt, phospho-PI3-K, and phospho-JNK. (**B**) Effects of TanIIA on ERK 1/2, Akt, PI3-K, and JNK phosphorylation in orbital fibroblasts of normal controls. A specific ERK kinase inhibitor, PD98059, significantly blocked the phosphorylation of ERK 1/2 in GO orbital fibroblasts pretreated with 10 μM TanIIA for 16 hours. (**C**) When cells were pretreated with PD98059, TanIIA-induced HO-1 was also significantly inhibited. The data in the column are the mean relative density ratios ± SD. Experiments were performed in triplicate with cells from three different donors. Primary orbital cells at passages 1 to 5 were used. *$P < 0.001$ versus untreated control differentiated cells.

Effect of TanIIA on Adipogenesis in Orbital Fibroblasts

To examine whether TanIIA had any suppressive effects on adipogenesis, it was added at day 1 into adipogenic medium, including rosiglitazone, and continued for the 10-day differentiation period, being replaced whenever media was replaced. High-power (×200, ×400) microscopic examination of Oil Red O staining showed that TanIIA dose-dependently decreased the size and number of intracytoplasmic lipid droplets in cells treated with or without rosiglitazone (Fig. 6A). Preadipocyte fibroblasts that did not convert into adipocytes were uniform in size and stellate in shape, which maintained viable fibroblastic morphology. To quantitatively evaluate adipocyte differentiation, the optical density of stained cell lysates was measured and TanIIA-treated cells showed significantly decreased absorbance at 490 nm in a dose-dependent manner ($P < 0.01$; Fig. 6B).

Western blot analysis was performed to investigate the effect of TanIIA on the expression of the adipogenic

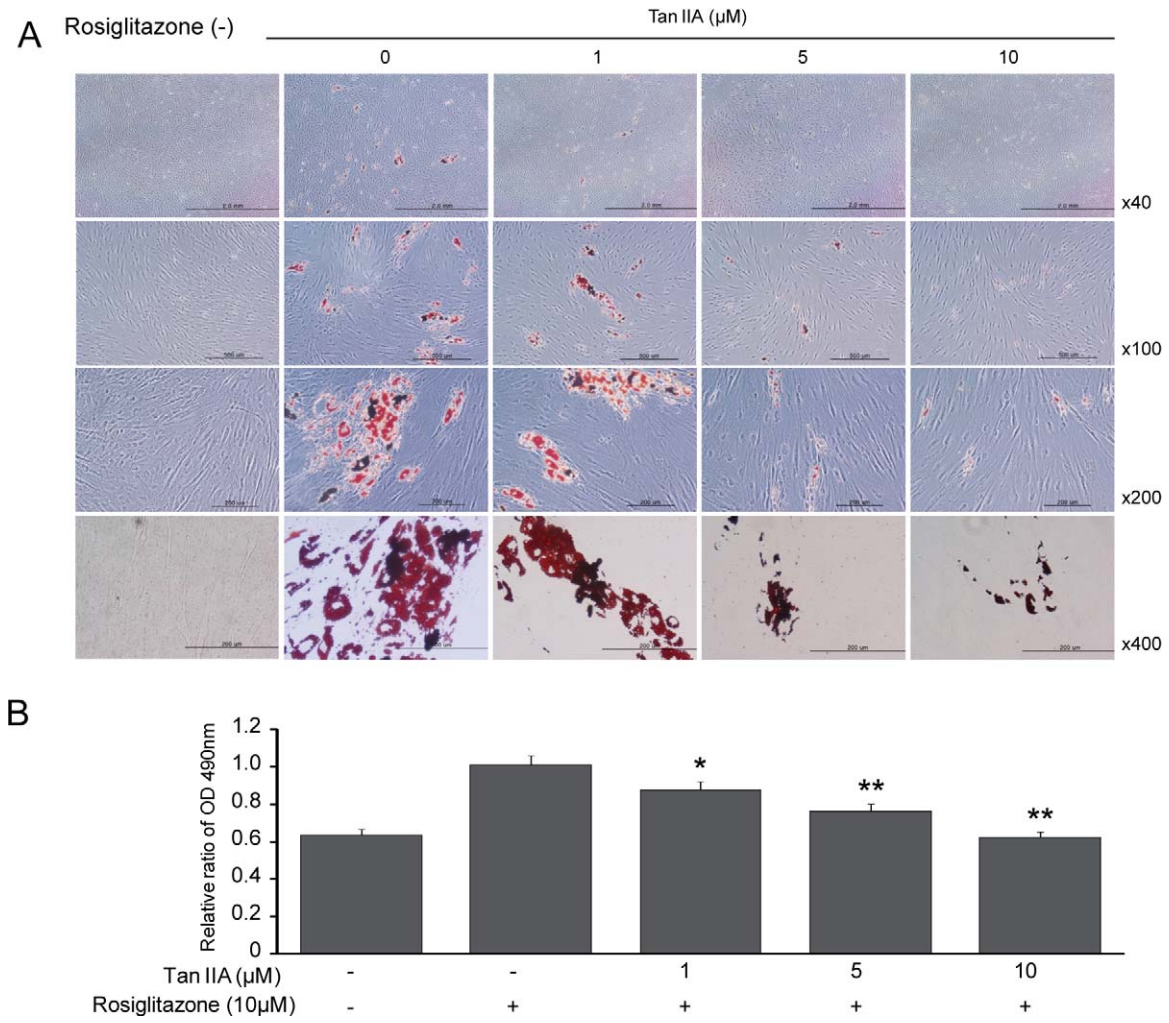

Figure 6. Effect of TanIIA on adipogenesis in GO orbital fibroblasts. (**A**) Microscopic examination of Oil Red O–stained cultures exposed to TanIIA in the adipogenesis of GO orbital fibroblasts. TanIIA treatment on the first day of initiation of 10-day adipogenesis in adipogenic media not containing rosiglitazone, or rosiglitazone and various concentrations (0–10 μM) of TanIIA. Cells at passages 1 to 5 were used. Cells were fixed with 10% formalin for 1 hour and then stained with Oil Red O to measure lipid accumulation and examined microscopically (×40, ×100, ×200, ×400). (**B**) Cell-bound Oil Red O was solubilized with 100% isopropanol and the optical density of the solution was measured at 490 nm to obtain a quantitative assessment of adipogenesis. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from three different donors, and the results were normalized to the absorbance of untreated control differentiated cells. Primary orbital cells at passages 1 to 5 were used. Results are expressed as percentage of untreated control values, presented as mean ± SD (in contrast to cells not treated with TanIIA, $^{*}P < 0.01$, $^{**}P < 0.001$).

transcription regulators PPARγ, C/EBP α, and C/EBP β. The protein levels of PPARγ and C/EBPα were significantly attenuated when treated with 5 to 10 μM TanIIA ($P < 0.01$; Fig. 7A).

When intracellular ROS levels were measured on days 0, 1, 4, 7, and 10 of adipocyte differentiation in GO orbital fibroblasts, the ROS levels were all significantly lower in differentiating cells treated with 10 μM TanIIA on days 4, 7, and 10 compared with cells without TanIIA treatment (all $P < 0.01$; Fig. 7B).

Discussion

In this study, we investigated the therapeutic efficacy and the related molecular mechanisms of TanIIA in the pathogenesis of GO in orbital fibroblasts. We found that TanIIA exhibited significant suppressive effects on inflammation, oxidative stress, and adipogenesis, all of which were the major pathologic mechanisms associated with the development of GO.

Tanshinone IIA, a lipophilic diterpene, has been reported to show unique biological properties that include anti-inflammatory,[11–13] antioxidant,[23–25] and antiadipogenic[15,16] activities. In the present investigation, we observed that TanIIA inhibited the IL-1β induction of the mRNA expression of proinflammatory cytokines in a dose-dependent manner. Tanshinone IIA has been reported to decrease the production of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1) by neutrophils, astrocytes, and macrophages.[26,27] Different mechanisms are involved in the anti-inflammatory action of TanIIA in many cell systems, as it targets multiple intracellular signaling pathways such as nuclear factor-κB activation and MAPK family phosphorylation.[13,26–28]

There is evidence that oxidative stress plays an important role in the pathogenesis of GO.[29–31] Superoxide radicals stimulate orbital fibroblasts to proliferate and to produce glycosaminoglycan[32] and H_2O_2 induces the expression of HLA-

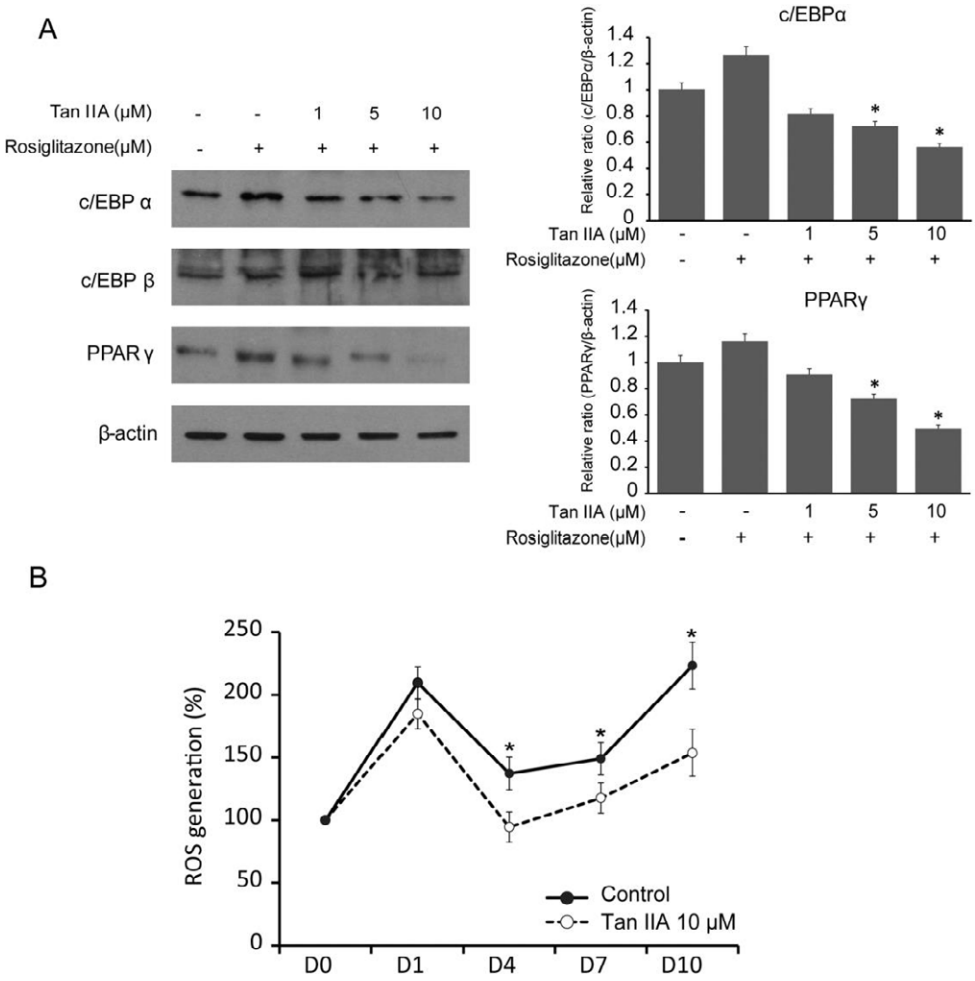

Figure 7. Effect of TanIIA on the expression of adipogenic transcriptional regulators in GO orbital fibroblasts and intracellular ROS in differentiating orbital fibroblasts during adipogenesis. (**A**) Tanshinone IIA (5–10 μM) treatment for the entire 10-day differentiation period in adipogenic media. After 10 days, cell lysates were subjected to Western blot analysis of PPARγ, C/EBP α, and C/EBP β protein expression. Quantification by densitometry, normalized to the β-actin level in the same sample, is shown for PPARγ and C/EBP α. The data in the column are the mean relative density ratios ± SD. (**B**) Confluent fibroblasts from GO patients were subjected to a differentiation protocol that included adipogenic supplements for 10 days. To determine the suppressive effect of TanIIA on adipogenesis, 10 μM TanIIA was also added during the entire 10-day differentiation period. Reactive oxygen species were measured by flow cytometry H_2DCFDA on days 0, 1, 4, 7, and 10 of adipogenesis. The results are expressed as percentages of the untreated control values, and presented as means ± SD. The assays were performed at least three times with cells from three different GO donors. Primary orbital cells at passages 1 to 5 were used. Differences between treated and untreated cells are indicated (in contrast to cells not treated with TanIIA, $^*P < 0.01$).

DR and heat shock protein 72.[33] Smoking is the strongest known environmental factor stimulating the occurrence and aggravation of GO by enhancing the generation of ROS and reducing antioxidant production.[34] In our study, TanIIA significantly inhibited CSE or H_2O_2-induced ROS generation in a dose-dependent manner in orbital fibroblasts from GO. Tanshinone IIA is reported to be a natural antioxidant and is known to protect various kinds of cells. Previous studies in vitro have revealed the antioxidant action of TanIIA by attenuating intracellular ROS levels in neonatal rat cardiomyocytes.[35,36] In another study, TanIIA protected H9c2 cells via preserving mitochondrial function by reducing the excess production of mitochondrial superoxide, superoxide dismutase activity, intracellular nitric oxide, and calcium levels, and restoring cellular ATP contents.[37] In brain microvascular endothelial cells, TanIIA suppressed the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 by suppressing ROS production.[38]

To gain further insight into the mechanism of TanIIA on ROS reduction, we next examined the effect of TanIIA on the expression of HO-1, an important cytoprotective, anti-inflammatory, and antioxidant enzyme. The key enzyme in heme catabolism, HO-1 functions as a cytoprotective mechanism against inflammatory responses and ROS insults through the anti-inflammatory action of its metabolite, CO, and the antioxidant activities of another metabolite, bilirubin.[39] The induction of HO-1 is considered part of the generalized protective response to oxidative stress, as an active defense mechanism. Our data showed that TanIIA had a profound inductive effect on HO-1 protein expression in human orbital fibroblasts, especially in GO cells. To identify which signal cascade controlled the activation of HO-1 by TanIIA, we examined the effects of MAPK and PI3-K inhibitors on the TanIIA-mediated upregulation of HO-1 expression; the results suggest that TanIIA-induced HO-1 expression is dependent on the activation of ERK rather than JNK 1/2, p38 MAPK, and PI3-K. The blockade of the ERK pathway by PD98059 strongly attenuated the increase of HO-1 by TanIIA, implying the involvement of the ERK signaling pathway in this antioxidant action. In our cells from GO, the induction of HO-1 expression

occurred together with ROS reduction. This is consistent with the finding that induction of HO-1 by isorhamnetin, a 3′-O-methylated metabolite of quercetin,[40] flavonoid kaempferol,[41] and curcumin, an anti-inflammatory agent extracted from *Curcuma longae*,[42] leads to a reduction in ROS production in RAW 264.7 cells. Curcumin also was found to reduce ROS levels by upregulating HO-1 expression in human retinal pigment epithelial cells.[43] TanIIA might cause ROS reduction by upregulating HO-1 protein in GO orbital fibroblasts; however, we have not yet proved the causal relationship, which would be investigated in our future study.

Tanshinone IIA significantly inhibited adipogenesis through induction of apoptosis in 3T3-L1 preadipocytes and adipocytes.[15,44] Tanshinone IIA also was found to decrease the amount of intracellular triglycerides and glycerol-3-phosphate dehydrogenase activity in 3T3-L1 adipocytes.[44] Furthermore, TanIIA inhibits 3T3-L1 preadipocyte differentiation and the transcriptional activity of full-length PPARγ and PPARγ ligand-binding domains.[15] The effects of TanIIA are mediated through its property as a natural antagonist of PPARγ.[15] We have previously reported that adipogenesis in Graves' orbital fibroblasts was upregulated by the oxidants H_2O_2 and CSE, and that this was suppressed in a dose-dependent manner by an antioxidant, quercetin, in parallel with a reduction in ROS.[5] In our study, microscopic results with Oil Red O staining with quantification showed that TanIIA suppressed adipocyte differentiation in a dose-dependent manner, and also suppressed the expression of PPARγ and C/EBPα protein. Tanshinone IIA also suppressed ROS generation in differentiating orbital fibroblasts during adipogenesis. Together, these suggest that TanIIA may suppress adipogenesis through its antioxidant effects.

Overall, our findings suggest that Tan IIA has the potential for anti-inflammatory, antioxidant, and antiadipogenic therapy in orbital fibroblasts from GO. Although TanIIA shows promising results in many studies, these have the limitation of being in vitro studies. As the oral bioavailability of TanIIA is very low,[45] it has limitations with respect to use in patients. However, recent studies have shown that solid dispersions of TanIIA with silica nanoparticles or low-molecular-weight chitosan achieved complete dissolution, increased the absorption rate, maintained drug stability, and showed improved oral bioavailability compared with TanIIA alone.[37,45] A study protocol for a randomized controlled trial has also been released to investigate the effect of sodium tanIIA sulfate and simvastatin on elevated serum levels of inflammatory markers in patients with coronary heart disease.[46] We believe the results in this study are noteworthy, as TanIIA can be used for the treatment of GO; further studies in humans using more developed types of TanIIA are needed to confirm these results.

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