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

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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 adiopogenesis-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 H2O2-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.

CONCLUSION. 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: adiopogenesis, 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.6 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 manage-
ment of moderate to severe GO is challenging, and often not
satisfactory. Previous treatments, such as high-dose glucocor-
ticoids and/or orbital radiotherapy, are mostly effective in
patients with severe and active eye disease.7 Soft tissue
inflammatory changes, recent-onset extraocular muscle in-
volvelement, 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 complica-
tions caused by systemic glucocorticoid therapy also should
not be ignored.

Tanshinone, isolated from *Salvia miltiorrhiza bunge*
danshen), has been widely used to treat coronary, cerebro-
vascular, and cardiovascular disease in traditional Chinese
medicine. Owing to its purported biological activity and lack of
serious side effects, as confirmed by pharmacological investiga-
tions 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 antifibrotic14 and
antiadipogenetic15,16 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), penicil-
lin, and gentamycin (Hyclone Laboratories, Inc., Logan, UT,
USA); 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bro-
mide (MTT), Oil Red O, and 2’-animo-3’-methoxyflavone
(PD98059) (Sigma-Aldrich Corp., St. Louis, MO, USA); bromo-
deoxyuridine (5-bromo-2-deoxyuridine [BrdU]) cell prolifera-
tion 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-extra-
cellular-regulated kinase (ERK) 1/2, anti-ERK 1/2, anti-phos-
pho-Akt, anti-phospho-phosphatidylinositol 3-kinase (PI3-K),
and anti-phospho-c-Jun 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 (4-2011-0590), 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. 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 genta-
mycin (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 × 104
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 540 Biokineitics 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 12,000g for 5 minutes to remove cell
derbris. Then, 100 µL of each sample was added to the substrate
solution and the absorbance at 490 nm was measured using a
microplate reader (VERsmax; Molecular Devices, Sunnyvale,
CA, USA). Activity of LDH was obtained by measuring the

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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 (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 (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, using commercially available, filtered cigarettes (Marlboro 20 class A cigarettes, made in Korea by Philip Morris Korea, Inc. (Seoul, Korea), and...
Probes, Inc., Eugene, OR, USA), an oxidant-sensitive fluorescent probe, as previously described, using the MTT assay. A dose of 2% CSE or 200 µM H₂O₂ was determined to be noncytotoxic.

Release of ROS was determined with 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes, Inc., Eugene, OR, USA), probed overnight with primary antibodies in Tris-buffered saline containing 8.0 mg of tar and 0.7 mg of nicotine). The noncytotoxic doses of CSE and H₂O₂ were determined as previously described, using the MTT assay. A dose of 2% CSE or 200 µM H₂O₂ was determined to be noncytotoxic.

Adipogenesis

Orbital fibroblasts were exposed to a differentiation protocol according to our previous reports, 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 pantethenic acid, 10 µg/mL transferrin, 0.2 nM T₃, 1 µM insulin (Boehringer-Mannheim, Manheim, Germany), 0.2 µM carboxyprostaglandin (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. 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 500 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 ×20 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

Differentially treated 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 Na₂VO₄, 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 12,000g 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).
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, nontoxic 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 H2O2-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 H2O2 for 1 hour was
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 \( \mu \)M) 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 \( \mu \)M) 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 \( \mu \)M 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 \( \mu \)M 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.
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
transcription regulators PPARγ, C/EBPα, and C/EBPβ. 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, antioxidant, and antiadipogenic 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. 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.

There is evidence that oxidative stress plays an important role in the pathogenesis of GO. Superoxide radicals stimulate orbital fibroblasts to proliferate and to produce glycosaminoglycan and H2O2 induces the expression of HLA-
DR and heat shock protein 72. Smoking is the strongest known environmental factor stimulating the occurrence and aggravation of GO by enhancing the generation of ROS and reducing antioxidant production. In our study, TanIIA significantly inhibited CSE or H₂O₂-induced ROS generation in a dose-dependent manner in orbital fibroblasts from GO. TanIIA 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. 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. In brain microvascular endothelial cells, TanIIA suppressed the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 by suppressing ROS production.

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. 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, flavonoid kaempferol, and curcumin, an anti-inflammatory agent extracted from Curcuma longa, 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. 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.

TanIIA significantly inhibited adipogenesis through induction of apoptosis in 3T3-L1 preadipocytes and adipocytes. TanIIA also was found to decrease the amount of intracellular triglycerides and glycerol-3-phosphate dehydrogenase activity in 3T3-L1 adipocytes. Furthermore, TanIIA inhibits 3T3-L1 preadipocyte differentiation and the transcriptional activity of full-length PPARγ and PPARγ ligand-binding domains. The effects of TanIIA are mediated through its property as a natural antagonist of PPARγ. We have previously reported that adipogenesis in Graves’ orbital fibroblasts was upregulated by the oxidants H2O2 and CSE, and that this was suppressed in a dose-dependent manner by an antioxidant, quercetin, in parallel with a reduction in ROS. 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. TanIIHone 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 II A 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, 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. A study protocol for a randomized controlled trial has also been released to investigate the effect of sodium tanIIA sulfate and an antioxidant, quercetin, in parallel with a reduction in ROS. and that this was suppressed in a dose-dependent manner by an antioxidant, quercetin via reduction in oxidative stress.

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