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The anti-inflammatory activities of Tanshinone IIA, an active component of TCM, are mediated by estrogen receptor activation and inhibition of iNOS

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ABSTRACT

Tanshinone IIA (Tan IIA) is a major compound extracted from a traditional herbal medicine *Salvia miltiorrhiza* BUNGE, which is used to treat cardiovascular diseases, cerebrovascular diseases and postmenopausal syndrome. It has also been shown to possess anti-inflammatory activity. Since Tan IIA has a similar structure to that of 17β-estradiol (E_2), the present study was undertaken to characterize the estrogenic activity of Tan IIA and to demonstrate a functional role of this activity in RAW 264.7 cells. In transient transfection assay, Tan IIA (10 μM) increases ERE-luciferase activity in an estrogen receptor (ER) subtype-dependent manner when either ERα or ERβ were co-expressed in Hela cells. In LPS-induced RAW 264.7 cells, Tan IIA exerts anti-inflammatory effects by inhibition of iNOS gene expression and NO production, as well as inhibition of inflammatory cytokine (IL-1β, IL-6, and TNF-α) expression via ER-dependent pathway. Therefore, it could serve as a potential selective estrogen receptor modulator (SERM) to treat inflammation-associated neurodegenerative and cardiovascular diseases without increasing the risk of breast cancer.

1. Introduction

Phytoestrogens are a diverse group of plant-derived compounds that structurally or functionally mimic mammalian estrogens and show potential benefits for human health [1]. It has been used to treat postmenopausal syndrome, breast cancer, osteoporosis and cardiovascular diseases, etc. Phytoestrogens are of biological interest because they exhibit both in vitro and in vivo weak estrogenic and antiestrogenic actions. An increase in phytoestrogen research over the past few decades has demonstrated the biological complexity of phytoestrogens, which belong to several different chemical classes and act through diverse mechanisms [2]. Phytoestrogens can bind to estrogen receptors (ERs) and mimic some actions of human estrogen through the activation or inactivation of certain genes. In the United States, the most commonly used alternative herbal medicines for estrogen replacement are soy, black cohosh, dong quai, and ginseng. *Radix Salviae Miltiorrhizae* (RSM) has been used traditionally for treating postmenopausal syndrome. It has been reported to exert estrogenic activity [3–5].

Tan IIA is one of the major compounds extracted from RSM, a well-known traditional Chinese herbal medicine (Danshen). It has been used in traditional Chinese medicinal preparations for treating cardiovascular [6,7] and cerebrovascular diseases [8,9]. Tan IIA has been observed to possess antioxidant [10], anti-inflammatory [11], as well as cytotoxic properties to multiple types of human cancer cells [12,13]. We found that anti-proliferative effect of Tanshinone in vascular smooth muscle cells (VSMCs) was associated with ERK1/2 signaling pathway [14].

The biological functions of estrogen in target tissues are mediated mainly by nuclear ERs that function as ligand-activated transcription factors to regulate gene expression. Two types of ERs, ERα and ERβ, have been identified [15]. Since ERα and ERβ can form homodimers or heterodimers, it is suggested that ERs may function through three different dimeric states, and it is possible that the different dimers could be activated by selective ligands. The existed difference in agonistic and antagonistic activation of ERα and ERβ by ligand may be due to tissue- or cell type-selectivity [16]. Certain natural hormones or phytochemicals are being shown to possess differential affinity and transactivation of ERα versus ERβ [17].

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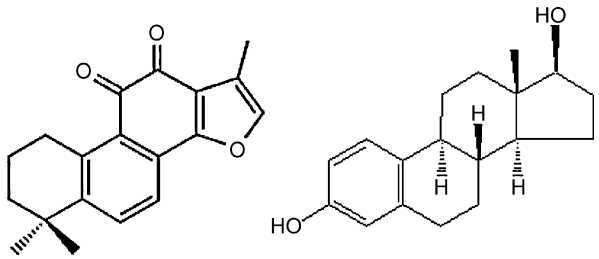

Tan IIA 17β-estradiol

Fig. 1. The molecular structure of Tan IIA and 17β-estradiol.

Since Tan IIA shares a similar structure with 17-β-estrodiol (E_2, Fig. 1), we postulated if it has estrogenic activities in ER positive (MCF-7) and immune (RAW 264.7) cells. We found that Tan IIA enhanced transcriptional activity of ERα and ERβ in transient transfection assay. Its estrogen-like effects differ in cells depending on their ERα and ERβ expression status. In LPS activated RAW 264.7 cells, Tan IIA exerts anti-inflammatory effects by inhibiting NO and inflammatory cytokine mRNA expression and production via ER-dependent pathway.

2. Materials and methods

All reagents were purchased from Sigma–Aldrich (St. Louis, USA) unless otherwise stated. Tan IIA was purchased from Chinese Institute for Drug and Biological Product Control (Beijing, China), Charcoal Dextran-stripped FBS (CD-FBS) was purchased from Biological Industries (Kibbutz Beit Haeme of Israel). ICI 182,780 was purchased from Tocris Bioscience (Ellisville, USA). E_2, ICI, and Tan IIA were dissolved in DMSO and further diluted in cell culture medium so that the final DMSO concentration did not exceed 0.1% (v/v).

2.1. Cell culture

Hela cells and Raw 264.7 cells were obtained from the American Type Culture Collection and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS, Hyclone, USA), 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1 mM pyruvate, 100 units penicillin/mL and 100 mg streptomycin/mL in an incubator containing humidified CO_2 (5%) at 37 °C. Prior to the beginning of each experiment, all cells were grown in estrogen-free medium for at least 1 day.

2.2. Transfection and reporter gene assay

Mammalian vectors expressing ERα and ERβ (pERα or pERβ) were gifts from Dr. Karas (Tufts Medical Center, Boston, USA). The luciferase reporter plasmid, pERE-TK-Luc, carrying 3× vitellogenin ERE was kindly provided by Dr. Donald MacDonald (Duke University, Durham, USA). Hela cells were plated in 96-well plates at a density of 8×10^3 cells/well in 5% CD-FBS. After attachment and growth for 24 h, the cells were co-transfected with the reporter plasmid pERE-TK-Luc with either ERα or ERβ expression plasmids. A control plasmid, pRL-TK, which contains a Renilla luciferase gene for normalizing transfection efficiency, was also co-transfected. Transfection was carried out for 6 h in serum-free, antibiotic-free DMEM medium using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instructions. Cells were treated with individual test compounds for 24 h following transfection. At the end, cells from each condition were lysed and divided into two 96-well plates for luciferase and renilla activity determination using a Topcount NXT luminescence counter (Packard Instrument Company, CT). Experiments were performed at least three times and the data were expressed as units of firefly luciferase activities normalized to the renilla luciferase control activities from individual wells.

Table 1
Primer sequences for IL-Iβ, IL-6, TNF-α, iNOS and β-actin used for quantitative real-time RT-PCR analysis.

Gene		Primer sequence(5′–3′)
Mouse IL-Iβ	Sense	TGTGAAATGCCACCTTTTGA
	Antisense	CCTCTTCGACACCGTCGATG
Mouse IL-6	Sense	TCCAGTTGCCTTCTTGGGAC
	Antisense	GTGTAATTAAGCCTCCGACTTG
Mouse TNF-α	Sense	TTCTGTCTACTGAACTTCGGGGTGATCGGTCC
	Antisense	GTATGAGATAGCAAATCGGATGACGGTGTGGG
Mouse iNOS	Sense	GGCAGCCTGTGAGACCTTTG
	Antisense	GCATTGGAAGTGAAGCGTTTC
Mouse β-actin	Sense	AGAGGGAAATCGTGCGTGAC
	Antisense	CAATAGTGATGACCTGGCCGT

2.3. Microwell E-Screen bioassay

MCF-7 cells were seeded into a 24-well plate (1×10^4 cells) in 5% FBS. The next day new medium containing 1% CD-FBS was replaced and various concentrations of Tan IIA were added to each well. Incubation continued for 5 days with changes of Tan IIA containing medium every 2 days. Cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [18]. All experiments were performed in triplicate.

2.4. Quantitative real-time RT-PCR analysis

Cells were harvested by centrifugation and washed twice in PBS. Total RNA was isolated from the cells using Trizol reagent [19] according to manufacturer's instructions. The mRNA expression of pS2, IL-1β, IL-6, TNF-α and iNOS were assessed by quantitative real-time PCR analysis. PCR reaction was performed in a 25 μl total volume including: 12.5 μl of 2× SYBR® Green RT-PCR master mix (Invitrogen, USA), 1 μg of cDNA template, and 0.2 μM each of gene-specific primers designed according to published literature (Table 1). Amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and different annealing temperature (55–60 °C). The mRNA expression levels were normalized to the expression of a housekeeping gene, HPRT or β-actin.

2.5. Measurement of nitrite concentration

The nitrite concentration in culture medium was measured using colorimetric assay based on the Griess reaction, according to the manufacture's protocol (Promega, USA).

2.6. Measurement of cytokine production

To investigate the inhibitory effects of Tan IIA on TNF-α, IL-1β, IL-6 and iNOS production in LPS-stimulated RAW 264.7 cells, cells (1×10^6) were incubated with Tan IIA (0.1–10 μM) and LPS (1 μg/mL) in 24-well plates for 24 h. Supernatants were collected and the levels of TNF-α, IL-1β, IL-6 and iNOS were quantified by ELISA [20], respectively.

2.7. Statistical analysis

All values are presented as mean ± S.D. The data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's *t*-test for multiple comparisons. Values of $P < 0.05$ or $P < 0.01$ were considered to be statistically significant.

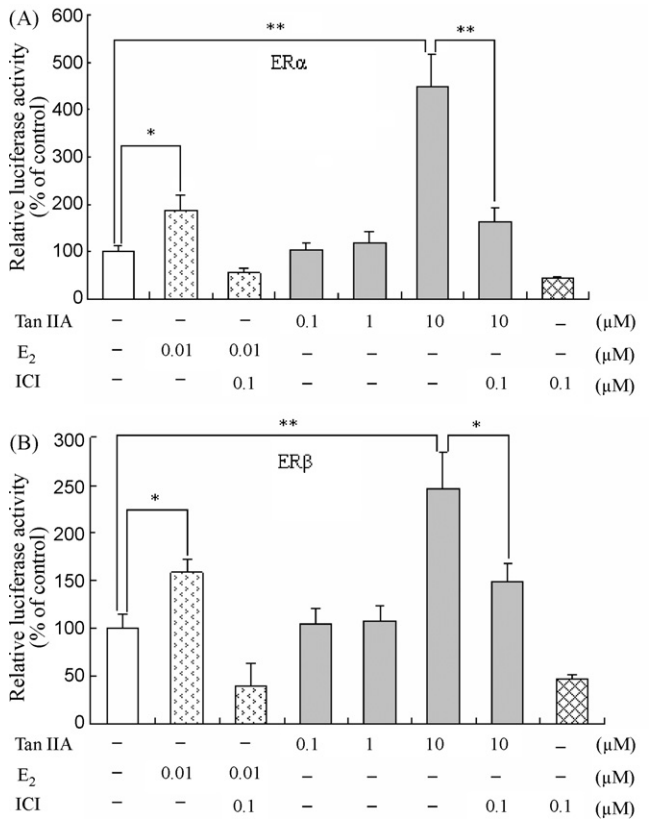

Fig. 2. Tan IIA enhances the basal ERα (A) and ERβ (B) transcriptional activity. Cells were transiently co-transfected with pERE-luc and pERα (A) or pERβ (B) by lipofection and treated with Tan IIA (0.1–10 μM) for 24 h and assayed for luciferase activity. E_2 (0.01 μM) was used as positive control. Transfections were performed more than three times. Data are displayed as mean percentage of vehicle control ± S.D. for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

3. Results

3.1. Tan IIA activates ERα and ERβ transcription in transient transfected Hela cells

The estrogen-like activity of Tan IIA was first observed by luciferase reporter gene technique. When Hela cells were co-transfected with pERE-luc and pERα or pERβ, E_2 (0.01 μM) increased the ERα or ERβ transactivation of the reporter gene significantly. Tan IIA (10 μM) also increased the ERα or ERβ transactivation significantly (Fig. 2A and B). Treatment of transfected cells with ICI (0.1 μM) alone or in combination with E_2 (0.01 μM) antagonized the relative luciferase units (RLU) to a level below the background activity.

3.2. Tan IIA inhibits the production of NO in LPS activated RAW 264.7 macrophages

To examine the effects of Tan IIA on inflammatory response, the estrogen receptor-positive murine monocytic line RAW 264.7 cells were used [21]. When the RAW 264.7 cells were incubated with 1 μg/mL of LPS for 24 h, the nitrite (NO_2^-) content in the medium increased 3.5-fold (Fig. 3). As expected, E_2 (0.01 μM) inhibited LPS-induced NO production by 35.5% and ICI (0.01 μM) completely reversed this inhibition (Fig. 3). Similarly, Tan IIA (0.1–10 μM) inhibited the LPS-induced NO production in RAW 264.7 cells by 26.5% at 1 μM and by 30.1% at 10 μM (Fig. 3). Tan IIA inhibition of iNOS was also likely mediated by ER since it was almost completely reversed by ICI (Fig. 3).

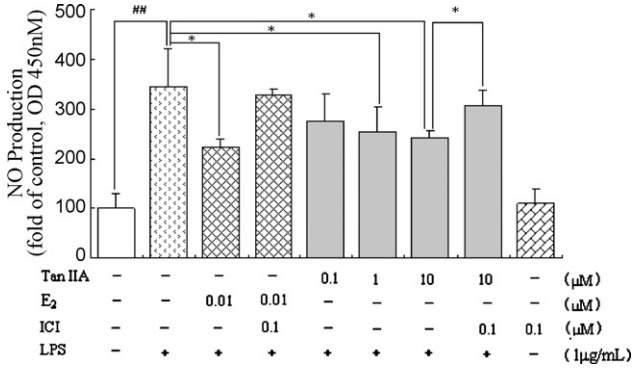

Fig. 3. Effect of Tan IIA on LPS-induced NO production in RAW 264.7cells. The cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h. NO production was determined by measuring the accumulation of nitrite in the medium. Data are displayed as mean percentage of vehicle control ± S.D. $^{\#\#}P < 0.01$, $^{*}P < 0.05$.

3.3. Tan IIA inhibits the production and expression of iNOS in LPS activated RAW 264.7 macrophages

To examine whether the inhibition of NO production by Tan IIA is due to a direct inhibition of inducible NOS, RAW 264.7 cells were incubated with various doses of Tan IIA (0.1–10 μM) and LPS (1 μg/mL) for 24 h. LPS induced mRNA expression and protein production of iNOS by 4.3-fold (Fig. 4A) and 5.5-fold (Fig. 4B), respectively. As previously reported, this increase was inhibited by 17β-estradiol (E_2, 0.01 μM) whereas ICI completely reversed the E_2 effect. Tan IIA exhibited a similar activity as E_2 although the

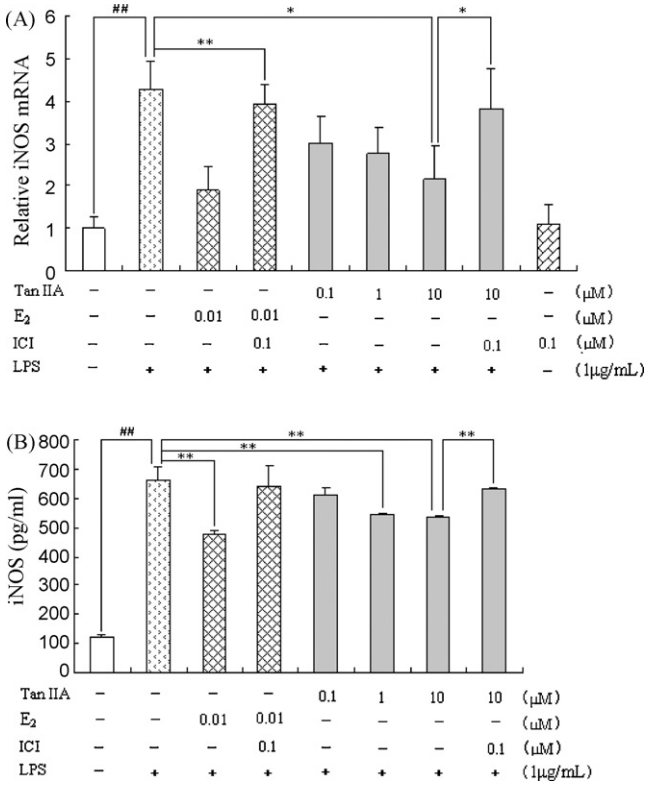

Fig. 4. Effect of Tan IIA on LPS-induced iNOS mRNA expression and production in RAW 264.7 cells. (A) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, total RNA were prepared and analyzed for iNOS mRNA expression by Quantitative real-time RT-PCR. (B) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, culture medium were used to assay iNOS production by ELISA. Data are displayed as mean fold of control ± S.D. $^{\#\#}P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$.

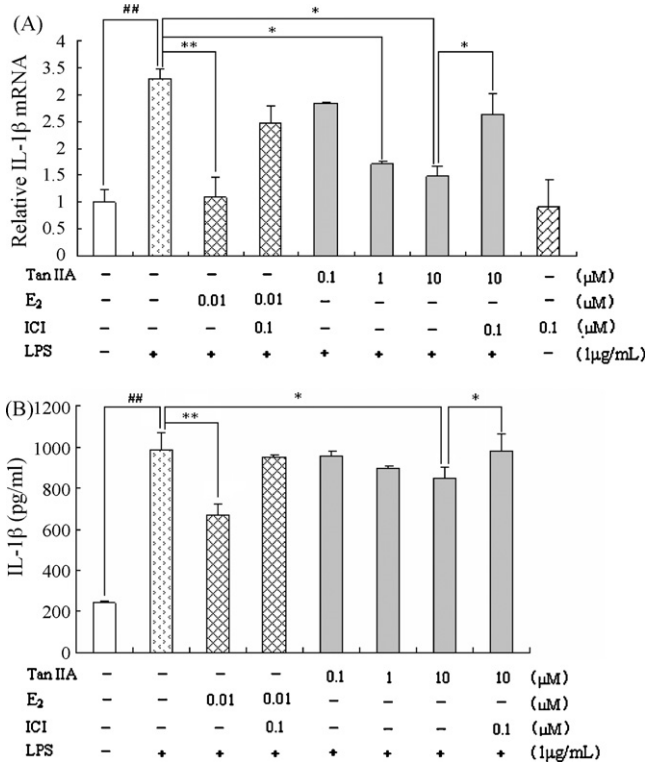

Fig. 5. Effect of Tan IIA on LPS-induced IL-1β mRNA expression and production in RAW 264.7 cells. (A) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, total RNA were prepared and analyzed for IL-1β mRNA expression by Quantitative real-time RT-PCR. (B) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, culture medium were used to assay IL-1β production by ELISA. Data are displayed as mean fold of control ± S.D. $^{\#\#}P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$.

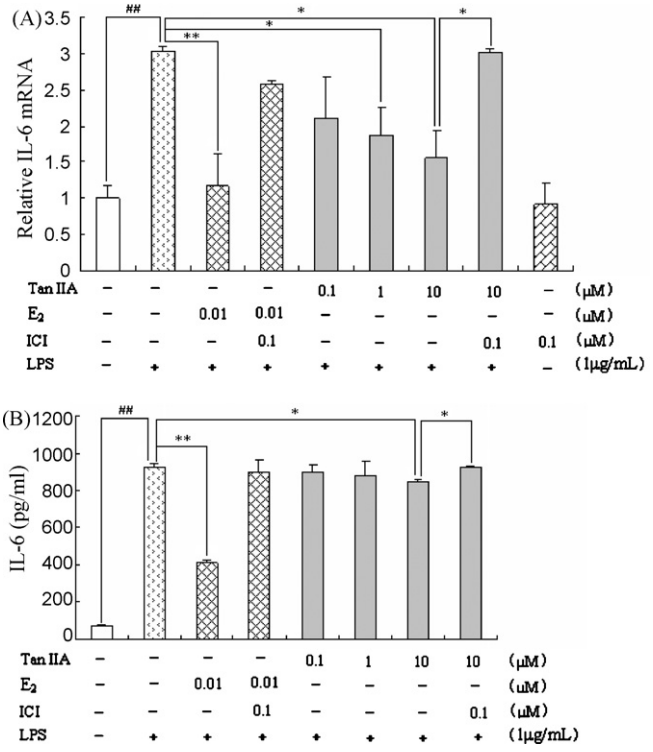

Fig. 6. Effect of Tan IIA on LPS-induced IL-6 mRNA expression and production in RAW 264.7 cells. (A) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, total RNA were prepared and analyzed for IL-6 mRNA expression by Quantitative real-time RT-PCR. (B) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, culture medium were used to assay IL-6 production by ELISA. Data are displayed as mean fold of control ± S.D. $^{\#\#}P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$.

concentration of Tan IIA required was higher (1–10 μM). Tan IIA at 10 μM significantly inhibited iNOS mRNA expression by 49.5% (Fig. 4A). Tan IIA at 1 μM and 10 μM significantly inhibited iNOS protein production (17.8% and 19.0%, respectively) (Fig. 4B). This inhibition was reversible by ICI (Fig. 4A and B).

3.4. Tan IIA inhibits the production and expression of IL-6, IL-10 and TNF-α in LPS activated RAW 264.7 macrophages

To examine whether Tan IIA could inhibit the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in RAW 264.7 cells, cells were incubated with various doses of Tan IIA (0.1–10 μM) and LPS (1 μg/mL) for 24 h. LPS induced mRNA expression and protein production of IL-1β by 3.3-fold (Fig. 5A) and 4.0-fold (Fig. 5B), respectively. Tan IIA at 1 μM and 10 μM significantly inhibited IL-1β mRNA expression by 55.2% and 48.2%, respectively (Fig. 5A). As control, E_2 inhibited LPS-induced IL-1β production whereas ICI reversed the E_2 inhibition. Tan IIA (10 μM) inhibited IL-1β production (13.6%) (Fig. 5B). This inhibition was reversible by ICI (Fig. 5A and B). LPS induced mRNA expression and protein production of IL-6 by 3.0-fold (Fig. 6A) and 12.6-fold (Fig. 6B), respectively. E_2 inhibited LPS-induced IL-6 production whereas ICI reversed the E_2 inhibition. Tan IIA at 1 μM and 10 μM significantly inhibited IL-6 mRNA expression by 38.2% and 48.8% (Fig. 6A), respectively and at 10 μM, it also inhibited IL-6 production by 8.7% (Fig. 6B). This inhibition was reversible by ICI (Fig. 6A and B). LPS induced mRNA expression and protein production of TNF-α by 2.5-fold (Fig. 7A) and 3.3-fold (Fig. 7B), respectively. E_2 inhibited LPS-induced TNF-α production whereas ICI reversed the E_2 inhibition. Tan IIA at 1 μM and 10 μM significantly inhibited TNF-α mRNA expression (43.4% and 47.1%, respectively) (Fig. 7A) and TNF-α protein production (9.0% and 15.1%, respectively) (Fig. 7B). This inhibition was reversible by ICI (Fig. 7A and B). The results suggest that Tan IIA's anti-inflammatory effect may be partly through inhibiting the production of NO, IL-1β, IL-6, TNF-α and iNOS and the expression of IL-1β, IL-6, TNF-α and iNOS mRNA expression.

4. Discussion

The rhizome of *Salvia miltiorrhiza Bunge*, also known as Danshen, is an important herb for promoting the circulation of blood and eliminating stasis in Chinese traditional medicine. *S. miltiorrhiza* has been commonly used to treat cardiovascular disease, inflammatory diseases and neurodegeneration diseases [8,9]. Experimental studies have shown that Tanshinone can inhibit leukocyte chemotaxis and therefore, may have therapeutic effect on acute and subacute inflammation [22,23]. In vitro and in vivo studies have shown that the anti-inflammatory mechanism of Tanshinone may be related to inhibition of inflammatory cell cytokine production and arachidonic acid metabolism [24]. Chemiluminescence assay was used to examine the inhibitory effect of Tanshinone on the production of oxygen-free radicals by human neutrophil. When preincubated with Tanshinone, chemiluminescence in neutrophils stimulated by fmLP (fmet-Leu-Phe) was markedly depressed. Similar results were observed when neutrophils were stimulator by PMA (phorbol myil-state acetate). These results suggest that inhibition of neutrophils to product oxygen-free radicals during inflammatory response may be another important anti-inflammatory mechanism of Tanshinone [25].

Tan IIA, a diterpene quinonic compound, is a major component of *S. miltiorrhiza*. Tan IIA has multiple pharmacological actions and has been used by traditional Chinese medicinal practitioners to treat

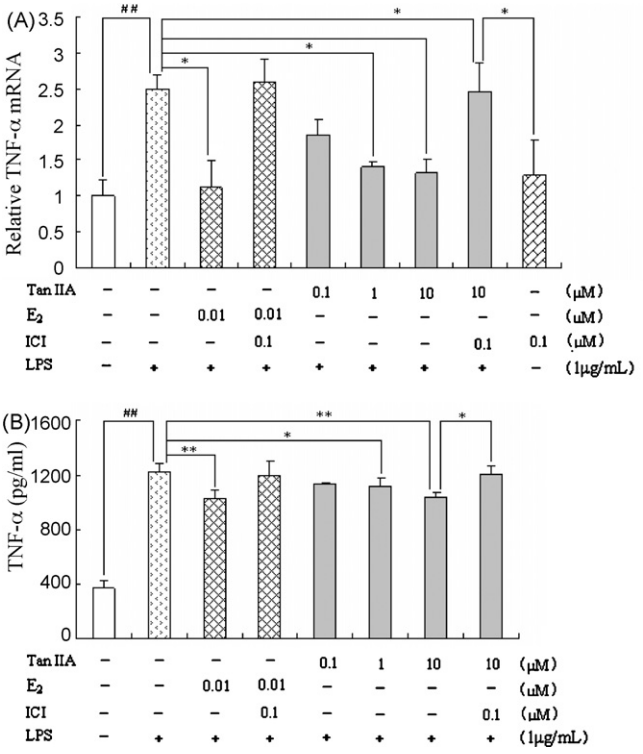

Fig. 7. Effect of Tan IIA on LPS-induced TNF-α mRNA expression and production in RAW 264.7 cells. (A) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, total RNA were prepared and analyzed for TNF-α mRNA expression by Quantitative real-time RT-PCR. (B) RAW 264.7 cells were treated with Tan IIA (0.1–10 μM) in the presence of 1 μg/mL LPS for 24 h, culture medium were used to assay TNF-α production by ELISA. Data are displayed as mean fold of control ± S.D. $^{\#\#}P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$.

cardiovascular diseases and cancers. The worldwide consumption of Danshen and its extracts is on the rise due to the increase in popularity of alternative medicine in the prevention and treatment of diseases. As a result, there is an increased interest in exploring the new therapeutic potentials of Tan IIA. Previous investigations showed that Tan IIA has antioxidant properties [10], anti-inflammatory properties [26] and cytotoxic activity against multiple cancer cell lines by inhibiting the proliferation of cancer cells [27,28]. Although numerous studies on the pharmacological actions of Tan IIA have been demonstrated, the details of the mechanism of the actions still need to be determined. Tan IIA has been used in clinics in China for treating postmenopausal syndrome. Since it shares a similar structure with E_2 (Fig. 1), we hypothesized that it may also possess an estrogenic activity. Our current study confirms that Tan IIA activate the transcription of both ERα and ERβ in transfected Hela cells, with a lower affinity compared to that of E_2.

Inflammation is involved in pathological process of many diseases, which are associated with increased production of inflammatory cytokines, such as ILs, and TNF-α [29]. The elevated cytokine level likely contributes to the increased incidence of inflammatory diseases, neurodegenerative diseases, menopause and cardiovascular diseases [30]. Our results confirmed that Tan IIA serves as a phytoestrogen and exerts anti-inflammatory effects by inhibiting NO, IL-1β, IL-6, TNF-α and iNOS production and mRNA expression. The effects are apparently mediated via an ER-dependent pathway in LPS activated RAW 264.7 cells since it could be blocked by ICI. The inhibition of cytokine production by Tan IIA may account for the antagonism of cell infiltration, suggesting that Tan IIA may be used as an anti-inflammatory drug against inflammatory disorders during menopause by limiting the early phases of macrophage infiltration. These results may explain the effectiveness of the traditional use of *S. miltiorrhiza* as an anti-inflammatory herbal medicine.

In summary, the present study indicates that the estrogen-like effects of Tan IIA are due, at least in part, to its ability to transactivate ERα or ERβ. Tan IIA exerts anti-inflammatory effects by inhibition of iNOS gene expression and NO production, as well as inhibition of inflammatory cytokine (IL-1β, IL-6, and TNF-α) expression via ER-dependent pathway. These findings provide new insights in understanding the complex actions of Tan IIA in immune response and suggest that it could serve as a potential selective estrogen receptor modulator (SERM) to treat inflammation-associated neurodegenerative and cardiovascular diseases without increasing the risk of breast cancer.

Acknowledgements

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