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# Nrf2 is involved in the effect of tanshinone IIA on intracellular redox status in human aortic smooth muscle cells☆

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ABSTRACT

Tanshinone IIA is the major antioxidant component in the traditional Chinese medicine *Salvia miltiorrhiza*. Transcription factor nuclear-factor-E2-related factor (Nrf2) regulates a battery of antioxidant response element (ARE)-regulated genes. The aim of this study was to determine the effect of tanshinone IIA on Nrf2 activation and intracellular redox status in human aortic smooth muscle cells. Tanshinone IIA potentiated tumor necrosis factor α (TNF-α)-mediated nuclear accumulation of Nrf2 and expression of ARE-related genes, while it reversed TNF-α-induced down-regulation of intracellular glutathione (GSH), NADPH and glucose 6-phosphate dehydrogenase (G6PDH) levels. Specific silence of Nrf2 by siRNA down-regulated tanshinone IIA-induced Nrf2 activation and increased of intracellular GSH, NADPH and G6PDH levels. Tanshinone IIA-induced Nrf2 activation was association with activation of ERK and PKB, which was prevented by treatment with PD098059 or wortmannin. Tanshinone IIA attenuated TNF-α, angiotensin II, $H_2O_2$-mediated reactive oxygen species (ROS) production. These results demonstrated that tanshinone IIA-induced Nrf2 activation is the major regulatory pathway of cytoprotective gene expression against oxidative stress via ERK and PKB signaling pathways.



## 1. Introduction

Reactive oxygen species (ROS) play a central role in vascular physiology and pathophysiology. They are produced by and affect all cell types in the vessel wall [1–5]. In the vasculature, ROS production has been observed in endothelial, smooth muscle, and adventitial cells. It has been shown to modulate cellular signaling cascades, gene expression, proliferation, apoptosis, and the vascular tone [1–4].

ROS-mediated cell injury contributes to the pathophysiology of cardiovascular disease and myocardial dysfunction. Protection against ROS-mediated cell injury requires maintenance of endogenous thiol pools, most importantly, reduced glutathione (GSH), by reduced nicotinamide adenine dinucleotide phosphate (NADPH). GSH provides the reducing equivalents necessary for the conversion of hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively, thereby preventing degradation to highly toxic free radicals, including hydroxyl and peroxyl radicals [6]. GSH also plays an important role in protection against oxidation of protein sulfhydryl groups. Glucose-6-phosphate dehydrogenase (G6PDH) is involved in the generation of NADPH and the maintenance of cellular redox balance [7]. Studies on antioxidative defense mechanisms of mammalian cells have demonstrated that G6PDH-generated NADPH is of crucial importance for protection tissues against oxidative injuries

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[8,9]. NADPH produced in the G6PDH reaction preserves cellular redox potential by providing reducing equivalents for glutathione reductase and thioredoxin reductase, which maintain glutathione and thioredoxin in the biologically active (reduced) state.

Nuclear-factor-E2-related factor (Nrf)-2 as a member of bZIP transcription factors is expressed in a variety of tissues. Transcriptional activation of antioxidant genes through an antioxidant response element (ARE) is largely dependent upon Nrf2. The functional ARE has been detected in a number of promoters of different genes, including glutathione S-transferase (GST), NAD(P)H: quinone oxidireductase (NQO), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL) and γ-glutamylcysteine synthetase (GCS) heavy and light subunits that play a role in defense against oxidative stress [10–12]. Nrf2 is normally sequestered in cytoplasm after binding to a repressor protein, Keap1, which is itself anchored to actin cytoskeleton [13]. Nrf2 might migrate to the nucleus after dissociation with Keap1 where it forms heterodimer with members of the small Maf family. Nrf2 binds to the ARE, and initiates phase 2 gene transcription. ARE-mediated gene expression plays a pivotal role in the cellular defense against the cellular damage caused by electrophiles and ROS [11].

Tanshinone IIA, the major component extracted from Radix *Salvia miltiorrhiza*, has numerous pharmacological activities such as antioxidant, prevention of angina pectoris and myocardial infarction [14]. The studies demonstrated that tanshinone IIA acted as an inducer of GSH-related phase II enzymes in human aortic smooth muscle cells (HASMCs), and tanshinone IIA modulated tumor necrosis factor α (TNF α)-induced oxidative stress in HASMCs through the detoxification system.

## 2. Materials and methods

### 2.1. Materials

Reduced glutathione, recombinant human TNF-α, angiotensin II (Ang II), and $H_2O_2$, were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Reverse Transcription System, the polymerase chain reaction (PCR) system were purchased from Promega (Madison, WI, USA). Rabbit anti-phospho-ERK1/2, -JNK, -PKB and -p38 antibodies, rabbit anti-Nrf2 (H-300) and rabbit anti-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059, U 0126, SP600125, SB203580, and wortmannin were purchased from Calbiochem (La Jolla, CA, USA). siRNA and scrambled sequences were purchased from Shanghai Genepharmacology company (Genepharma, Shanghai, China).

### 2.2. Cell cultures

Human aortic smooth muscle cells (HASMCs), medium 231 and SMC growth supplement were purchased from Cascade Biologics (Portland, Oregon, USA). HASMCs were grown in 231 medium with growth supplement (fetal bovine serum (5%, v/v final concentration), human basic fibroblast growth factor, human epidermal growth factor, and insulin) at 37 °C in a humidified 95% air/5% $CO_2$ atmosphere. For all experiments, HASMCs were grown to 80–90% confluence and made quiescent by starvation for 24 h. Tanshinone IIA, PD98059, U 0126, SP600125, SB203580, and wortmannin were added 30 min before treatment with TNF-α, $H_2O_2$ or Ang II.

### 2.3. Nrf2 siRNA knockdown

The siRNA target sequence for Nrf2 was 5′-AAG AGT ATG AGC TGG AAA AAC-3′) [15]. siRNA (50 nM) or scrambled control (50 nM) was transfected to HASMCs by Lipofectin (Invitrogene, Carlsbad, CA, USA) according to the manufacture's instructions. Briefly, cells were seeded in six-well plates and incubated overnight, then transfected with 50 nM siRNA for 12 h using 5 μL Lipofectin per well. The cells were cultured for another 48 h, then used for experiments.

### 2.4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HASMC cells using the Trizol Reagent and quantified by UV absorption at 260 and 280 nm. Total RNA (2 μg) was reverse-transcribed into cDNA using an oligo (dT) primer, and then amplified with specific primers. The following primers with the predicted size were used for amplication—*Nrf2*: (F) 5′-GCG ACG GAA AGA GTA TGA C-3′, (R) 5′-GTT GGC AGA TCC ACT GGT TT-3′; *NQO1*: (F) 5′-GGC TGG TTT GAG AGA GTG-3′, (R) 5′-GTC GGC TGG AAT GGA CTT G-3′; *HO-1*: (F) 5′-CAG CAC TAC GTA AAG CGT CTC C-3′, (R) 5′-AGT GCT GAT CTG GGA TTT TCC T-3′; *GCSL*: (F) 5′-CTT GAA TGA ATG GAG TTC CCA-3′, (R) 5′-TAC CTG TGC CCA CTG ATA CAG C-3′; *GCSH*: (F) 5′-CCA GTT CCT GCA CAT CTA CCA CGC-3′, (R) 5′-GCA GAA ATC ACT CCC CAG CGA C-3′; *GCLM*: (F) 5′-ACC TGG CCT CCT GCT GTG TG-3′, (R) 5′-GGT CGG TGA GCT GTG GGT GT-3′; *GCLC*: (F) 5′-ACA AGC ACC CCC GCT TCG GT-3′; *GAPDH*: (F) 5′- CGG AGT CAA CGG ATT TGG TCG TAT-3′, (R) 5′- AGC CTT CTC CAT GGT GGT GAA GAC-3′. The cycle number was determined from a linear amplification curve as being within the linear amplification range. The PCR conditions were as follows: 94 °C for 30 s, 55–60 °C for 30 s, and 30 s for 72 °C. Amplification products were visualized on 1.5% agarose gel and visualized by ethidium bromide staining and quantified densitometrically (GADPH as a loading control)using Quantity One software (Bio-Rad, Hercules, CA).

### 2.5. Preparation of nuclear extract and Nrf2 analysis

Cells were scraped into ice-cold lysis buffer (10 mM Hepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 0.5 mM DTT, and 0.4% NP-40), incubated for 10 min, and centrifuged at 14,000 × *g* for 3 min at 4 °C. The resulting nuclear pellet was resuspended in extraction buffer (20 mM HEPES (pH 8.0), 0.4 M NaCl, 1.0 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10% glycerol), kept on ice for 15 min, and centrifuged at 14,000 × *g* for 5 min at 4 °C. The supernatant containing the nuclear proteins was resolved by SDS-PAGE

and Nrf2 levels determined by western blotting using Nrf2 antibodies.

### 2.6. Western immunoblotting

Cells were lysed in 50 mmol/L Tris–HCl—150 mmol/L NaCl, pH 7.5, buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mmol/L NaF, 2 mmol/L $Na_3VO_4$, 10 mmol/L phenylmethylsulfonylfluoride, 150 nmol/L aprotinin, and 1 μmol/L leupeptin. Protein concentrations were measured with the Bio-Rad Protein Assay. Equivalent amounts of protein were electrophoresed in 10% SDS-PAGE. After transfer to nitrocellulose membranes, bands were visualized by reaction with primary antibody. In brief, membranes were blocked in 5% bovine serum albumin and probed with primary antibody for 1 h at room temperature. After secondary incubation in horseradish peroxidase-conjugated goat anti-mouse or goat-anti rabbit IgG antibody (1:2500) (Santa Cruz, CA), the immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL) from Pierce. Bands corresponding to different proteins were quantified by scanning of photographs and then digitalized and analyzed with the Bandscan software.

### 2.7. Measurement of glucose 6-phosphate dehydrogenase (G6PDH) activity

Cells were washed with fresh medium, detached with trypsin/EDTA, washed with PBS, resuspended at $0.1 \times 10^6$ cells/mL in 0.1 M Tris, 0.5 mmol/L EDTA, pH 8.0, and sonicated on ice with two 10 s bursts. Cell lysate was supplemented with 10 mmol/L $MgCl_2$ and 0.25 mmol/L $NADP^+$, and each measurement was performed on 1 mL of the reaction mixture. The most commonly used assays for G6PDH activity measure the rate of reduction of $NADP^+$ to NADPH when a cell lysate is incubated with glucose 6-phosphate. G6PDH activity was assayed by measuring the rate of NADPH production from 0.5 mmol/L $NADP^+$ and 2 mmol/L glucose-6-phosphate. Enzymatic activity was expressed as pmol $NADP^+$ reduced/min/mg cell protein [16].

### 2.8. Assay for cellular glutathione content

Intracellular GSH levels were determined by a glutathione reductase-coupled 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) assay. Briefly, each cell pellet was sonicated in 150 μL PBS. Lysates (20 μL) were mixed with 80 μL 2.5% metaphosphoric acid (MPA) on ice for 20 min and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant fraction was diluted 20-fold with assay buffer (100 mmol/L sodium phosphate plus 5 mmol/L EDTA, pH 7.5). In each well, 50 μL diluted samples were mixed with 50 μL 1.26 mM DTNB in assay buffer and 50 μL 2.5 U/mL glutathione reductase (in assay buffer). After 5 min incubation at room temperature, 50 μL 0.72 mmol/L NADPH in assay buffer were added to each well and the initial reaction rates were measured at 405 nm by the microtiter plate reader. Several concentrations of pure GSH were also assayed in the same plate to establish a calibration curve for calculation of GSH content in the samples. The concentration of GSH was expressed as nanomoles per milligram protein.

### 2.9. Measurement of NADPH levels

NADPH was measured by a method described by Stanton and co-workers [17]. In brief, the method is based on the fact that only NADH and NADPH (but not $NAD^+$ or $NADP^+$) affect absorbance at 340 nm. Cell extracts are separated into three aliquots (A1, A2 and A3). A1 is untreated, A2 is treated with G6PDH that converts all of the $NADP^+$ to NADPH, A3 is treated with glutathione reductase that converts all of the NADPH to $NADP^+$, and then the absorbance at 340 nm is measured. A1–A3 is the NADPH content of the extract.

### 2.10. Measurement of intracellular ROS levels

Intracellular ROS were assessed with the ROS-sensitive fluorophore DCFH-DA (Sigma) [18]. After reaching 80–90% confluence on a 96-well plate, cells were washed once with serum-free medium and then incubated with TNF-α in serum-free medium for the periods indicated in the figure legends. During the last 30 min of incubation, 10 μmol/L DCF was added to the culture. The fluorescence intensity was read directly from the culture plate at an emission wavelength of 535 nm and an excitation wavelength of 490 nm with a multiwell microplate reader.

### 2.11. Statistical analysis

Results are expressed as the means ± S.E.M. Statistical analysis of the data was done by one-way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison Test. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of tanshinone IIA on TNF-α-induced redox status in HASMCs

To clarify whether the effect of tanshinone IIA was related to the alteration of intracellular redox status, we examined intracellular GSH, G6PDH and NADPH levels in HASMCs. As shown in Fig. 1, intracellular GSH, G6PDH and NADPH levels was significantly down-regulated by treatment with TNF-α (10 ng/mL) for 24 h; pretreatment with tanshinone IIA for 30 min provided significant protection against down-regulation of intracellular GSH, G6PDH and NADPH levels caused by TNF-α.

### 3.2. Effects of tanshinone IIA on Nrf2 activation and ARE responsed genes in HASMCs

To determine the molecular details of intracellular GSH, G6PDH and NADPH levels elevation by tanshinone IIA, we focus on Nrf2, a transcription factor for ARE gene expression. After treatment with TNF-α for 24 h, nucleus Nrf2 levels were significantly elevated; tanshinone IIA potentiated TNF-α-induced Nrf2 activation in HASMCs (Fig. 2A). As shown in Fig. 2B, treatment of the cells with tanshinone IIA indeed enhanced the GCSL, GCSH, GCLC, GCLM, NQO1 and HO-1 mRNA expressions, all of which were reported to be regulated by the Nrf2-dependent pathway.

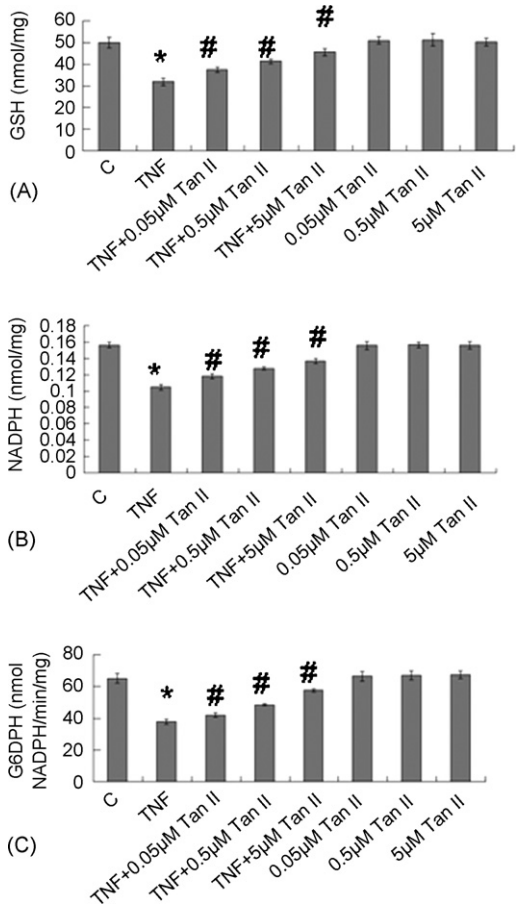


**Fig. 1 – Effects of tanshinone IIA on intracellular GSH, NADPH and G6PDH levels in HASMCs. HASMCs were treated with 0.05, 0.5, 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL) for 24 h. (A) Assays of intracellular GSH levels were performed as described under Section 2. (B) Assays of intracellular NADPH levels were performed as described under Section 2. (C) Assays of intracellular G6PDH levels were performed as described under Section 2. Results were given as mean ± S.E.M. of six independent experiments. $^{*}p < 0.05$ when compared with the control values. $^{\#}p < 0.05$ when compared with the TNF-α values.**

### 3.3. *Effects of siRNA Nrf2 on tanshinone IIA-induced Nrf2 activation and redox status in HASMCs*

We confirmed the role of Nrf2 in tanshinone IIA-induced alteration of redox status using small interfering RNAs (siRNAs) to inhibit the endogenous expression of Nrf2 in HASMCs. The transfection of HASMCs cells with an siRNA specific for Nrf2 greatly reduced Nrf2 mRNA expression (Fig. 3A). Similarly, transfection with the Nrf2-specific siRNA greatly reduced Nrf2 protein expression (Fig. 3B). By contrast, the scrambled control siRNA did not affect expression of either Nrf2 mRNA or Nrf2 protein as compared to endogenous levels in non-transfected cells (Fig. 3A and B). Tanshinone IIA-induced upregulation of Nrf2 mRNA and Nrf2 protein compared with transfection with the Nrf2-specific siRNA alone.

Transfection with Nrf2-siRNA almost completely diminished the induction levels of GSH, G6PDH and NADPH, but not with the scrambled control (Fig. 3C–E). Tanshinone IIA provided significant protection against down-regulation of intracellular GSH, G6PDH and NADPH levels caused by transfection with the Nrf2-specific siRNA.

### 3.4. *Effects of MAPKs and PI3K/PKB signaling pathways on tanshinone IIA-induced Nrf2 activation and redox status in HASMCs*

PI3K/PKB and three major MAPKs: ERK1/2, p38, and JNK play an important role in mediating cellular effects in HASMCs. We determined whether these pathways were involved in the

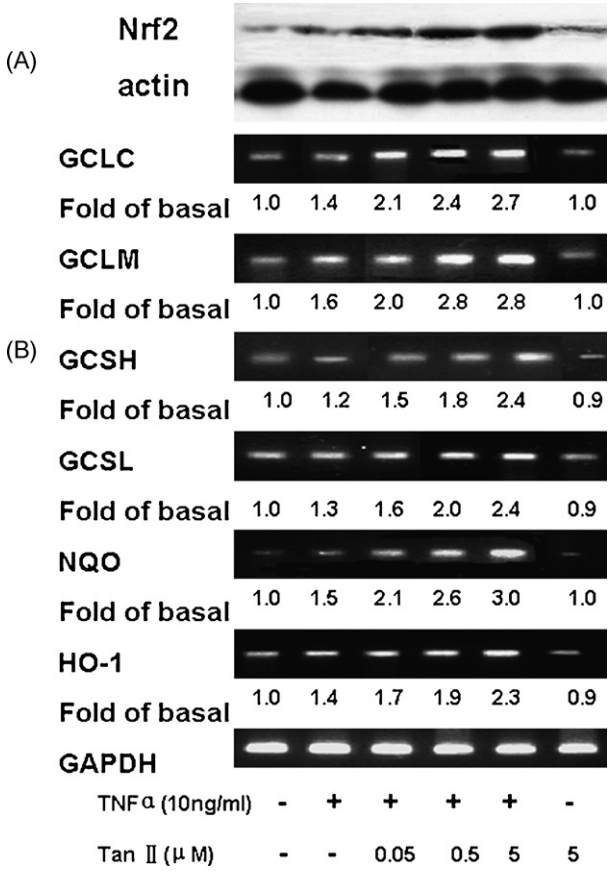


**Fig. 2 – Effects of tanshinone IIA on Nrf2 nuclear translocation and antioxidant response element (ARE)-mediated gene expression in HASMCs. (A) HASMCs were treated with 0.05, 0.5, 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL) for 1 h. Nrf2 proteins in nucleus were detected by Western blot. The blots shown were representative of three independent experiments with similar results. (B) HASMCs were treated with 0.05, 0.5, 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL) for 24 h. Total RNA was harvested and RT-PCR was performed and products were resolved by gel electrophoresis. RT-PCR products were normalized to GAPDH products. The intensity of PCR product bands shown were quantitated by scanning densitometry. The results shown were representative of three independent experiments with similar results.**

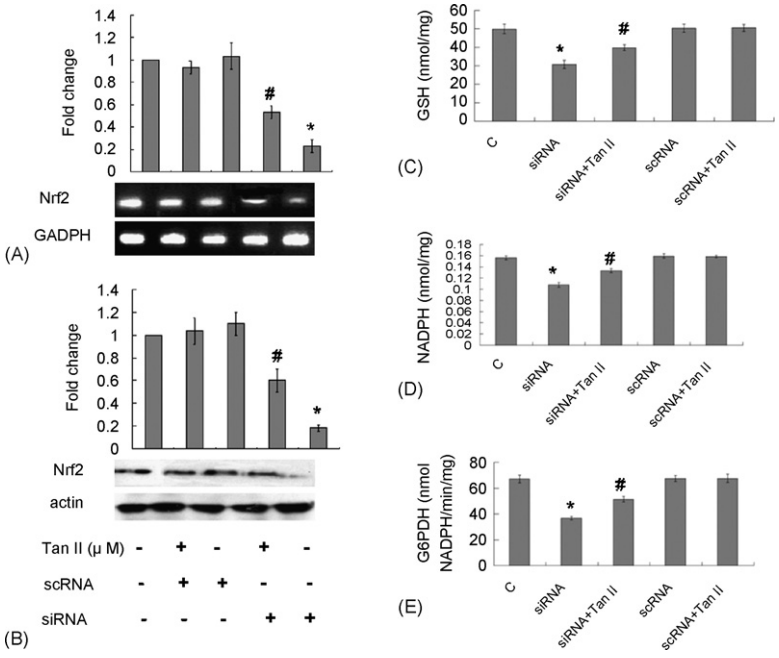


**Fig. 3 – Effects of siRNA Nrf2 on tanshinone IIA regulation of intracellular GSH, NADPH and G6PDH levels in HASMCs. HASMCs were transfected with 50 nM siRNA or 50 nM siRNA as described under Section 2, and then treated with 5 μM tanshinone IIA for 24 h. (A) Nrf2 RNA levels and (B) Nrf2 proteins in nucleus were detected by RT-PCR and Western blot, respectively. Results are shown as mean ± S.E.M. of three independent experiments. (C) Assays of intracellular GSH levels were performed as described under Section 2. (D) Assays of intracellular NADPH levels were performed as described under Section 2. (E) Assays of intracellular G6PDH levels were performed as described under Section 2. Results were given as mean ± S.E.M. of six independent experiments. $^{*}p < 0.05$ when compared with the control values. $^{\#}p < 0.05$ when compared with the siRNA values.**

tanshinone IIA's cytoprotective effect. In HASMCs cells, ERK1/2 and PKB phosphorylation peaked at 30 min after treatment with TNF-α stimulation and detected slowly over 1 h; phosphorylation of p38 and JNK also peaked at 30 min and returned quickly to basal levels (data not shown). In HASMCs pretreated with tanshinone IIA, TNF-α-induced ERK1/2 and PKB phosphorylation were enhanced and TNF-α-induced p38 and JNK phosphorylation were unaffected (Fig. 4A).

To test whether MAPKs and PI3K/PKB signaling pathways were involved in tanshinone IIA-induced Nrf2 activation, cells were pretreated with wortmannin, a PI3K inhibitor; SP600125, a JNK inhibitor; SB 203580, a p38 inhibitor or PD98059, a MEK inhibitor. PD98059 and wortmannin, but neither SP600125 nor SB 203580 inhibited tanshinone IIA pretreatment with TNF-α-induced the nuclear accumulation of Nrf2 protein (Fig. 4B) and increase intracellular GSH (Fig. 4C), G6PDH (Fig. 4D), NADPH (Fig. 4E) levels.

### 3.5. *Effects of tanshinone IIA on Ang II and $H_2O_2$ induced Nrf2 activation and intracellular redox status in HASMCs*

We next studied whether tanshinone IIA could affect Nrf2 activation and intracellular redox status induced by $H_2O_2$ or Ang II, an agonist, which stimulates NADPH oxidase-dependent ROS generation in HASMCs. Tanshinone IIA significantly enhanced $H_2O_2$ (100 μM) and Ang II (100 nM)-induced Nrf2 activation (Fig. 5A); while tanshinone IIA significantly reversed $H_2O_2$ and Ang II-induced down-regulation of intracellular GSH (Fig. 5B), G6PDH (Fig. 5C) and NADPH (Fig. 5D) levels.

### 3.6. *Effects of tanshinone IIA on reactive oxygen species (ROS) production induced by TNF-α, Ang II or $H_2O_2$*

To examine whether tanshinone IIA could affect ROS production induced by TNF-α, $H_2O_2$ or Ang II, we measured intracellular ROS levels using dichlorofluorescein diacetate (DCFH-DA). As shown in Fig. 6, $H_2O_2$ (100 μM) and Ang II (100 nM) significantly enhanced ROS production, while tanshinone IIA (5 μM) significantly reversed TNF-α, $H_2O_2$ and Ang II-induced ROS production.

## 4. Discussion

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide ($H_2O_2$), nitric oxide, and peroxynitrite, are important signaling molecules that regulate vascular tone and structure. Redox metabolism corresponds to a complex interacting network involving the generation of ROS, and enzymatic and non-enzymatic cellular antioxidant defenses [19]. Any small and transient disturbance of this balance induces redox signaling, which can act on several signaling transduction pathways, or enzyme and transcription factor activities. In contrast, when antioxidant defenses are

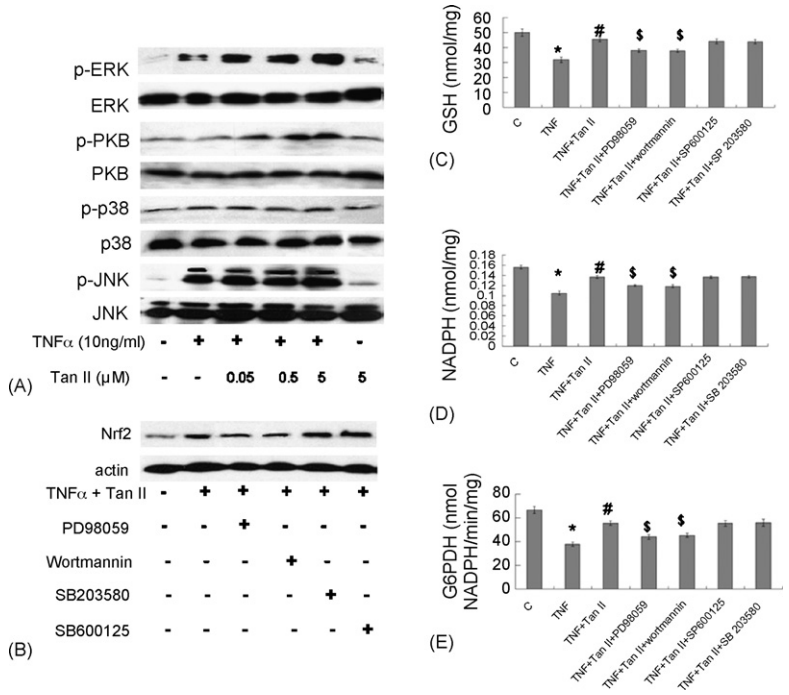


**Fig. 4 – ERK and PKB were involved in tanshinone IIA induction of Nrf2 nuclear translocation and GSH, NADPH, G6PDH levels in HASMCs. (A) Tanshinone IIA potentiated TNF-α-induced ERK and PKB protein phosphorylation in HASMCs. HASMCs were treated with 0.05, 0.5, 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL) for 30 min. Phosphorylated ERK (p-ERK), ERK, p-PKB, PKB, phosphorylated p38 (p-p38), p38, phosphorylated JNK, and JNK were detected by Western blot. The results shown were representative of three independent experiments with similar results. (B) HASMCs were treated with 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL) for 24 h in the absence or presence of 50 μM PD98059, 100 nM wortmannin, 10 μM SB203580, or, or 10 μM SP900125. All inhibitors were added into the medium 1 h before addition of tanshinone IIA. Inhibitors were dissolved in DMSO (0.1%, v/v final concentration); controls contained 0.1%, v/v DMSO. Nrf2 proteins in nucleus were detected by Western blot. The blots shown were representative of three independent experiments with similar results. (C) Assays of intracellular GSH levels were performed as described under Section 2. (D) Assays of intracellular NADPH levels were performed as described under Materials and methods. (E) Assays of intracellular G6PDH levels were performed as described under Section 2. Results were given as mean ± S.E.M. of six independent experiments. $^{*}p < 0.05$ when compared with the control values. $^{\#}p < 0.05$ when compared with the TNF-α values. $^{\$}p < 0.05$ when compared with the TNF-α and Tan II values.**

chronically overwhelmed, this phenomena results in an oxidative stress in which free radicals may exert their deleterious effects [19]. In the present study, we demonstrated that tanshinone IIA, a major antioxidant component from traditional Chinese medicine *Salvia miltriorhiza*, induced nuclear accumulation of Nrf2 protein levels and increased intracellular GSH, G6PDH and NADPH levels and provided cytoprotective effects in HASMCs.

Cellular redox potential is largely determined by GSH that is one of the most intensively studied intracellular compounds due to its critical role in cell biochemistry and physiology [20]. This tripeptide is present in a reduced form (GSH) and in two oxidized species: GSH disulphide (GSSG) and GSH mixed disulphide with protein thiols (GS-R). Maintaining an optimal GSH: GSSG ratio in the cell is critical to cell survival, hence, tight regulation of the system is imperative [21]. GSH is required for the maintenance of the thiol redox status of the cell, protection against oxidative damage, detoxification of endogenous and exogenous reactive metals and electrophiles, storage and transport of cysteine, as well as for protein and DNA synthesis, cell cycle regulation and cell differentiation [22–24].

Glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme in the pentose phosphate pathway, is necessary for the synthesis of pentoses [7]. G6PDH is also involved in antioxidant defense by providing NADPH, a major intracellular reductant. NADPH is a cofactor in the glutathione (GSH) reductase catalyzed reaction to regenerate antioxidant GSH and it is also required in maintaining the antioxidant enzyme catalase in its active form [9]. Mouse embryonic stem cells with deleted G6PD gene fail to induce NADPH production in response to oxidative stress, and these results in decreased GSH/GSH disulfide ratio and cell death [25]. Even mild G6PDH deficiency produces a distortion of redox control and oxidative mutagenesis [26]. On the other hand, overexpression of G6PDH suppresses hydrogen peroxide-induced cell death and the protection is mediated through the induction of GSH production [8,9]. Previous studies have found that G6PDH gene expression was regulated by intracellular redox status. ROS/reactive nitrogen species (RNS), such as hydrogen peroxide

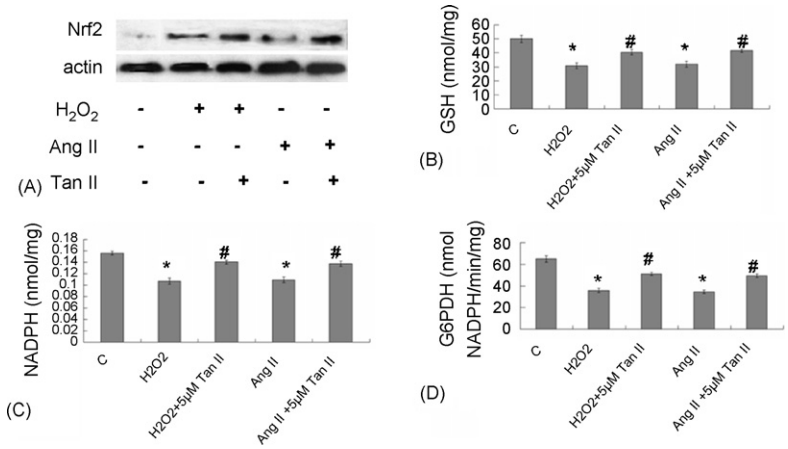


**Fig. 5 – Effects of tanshinone IIA on $H_2O_2$ or Ang II-induced Nrf2 nuclear translocation and intracellular GSH, NADPH and G6PDH levels in HASMCs. HASMCs were treated with 0.05, 0.5, 5 μM tanshinone IIA for 30 min before treatment with $H_2O_2$ (100 μM) or Ang II (100 nM) for 24 h. (A) Nrf2 proteins in nucleus were detected by Western blot. The blots shown were representative of three independent experiments with similar results. (B) Assays of intracellular GSH levels were performed as described under Section 2. (C) Assays of intracellular NADPH levels were performed as described under Section 2. (D) Assays of intracellular G6PDH levels were performed as described under Section 2. Results are given as mean ± S.E.M. of six independent experiments. $^{*}p < 0.05$ when compared with the control values. $^{\#}p < 0.05$ when compared with the $H_2O_2$ or Ang II values.**

and peroxynitrite, activate G6PDH as part of a cellular response against oxidative damage [8,9]. Our results demonstrated that the increase of intracellular G6PDH and NADPH levels was a cellular compensatory response to loss in GSH and the existence of a pro-oxidant state in HASMCs exposed to oxidative stress.

Recently, the role of the ARE in regulating the transcription of the two GCL subunits as well as other phase II detoxification enzymes has received a great deal of attention [27]. The transcriptional activation of these proteins is mediated at least in part by ARE. Transcriptional activation through the ARE is dependent upon the transcription factor Nrf2, a member of the Cap'n'Collar family of bZIP proteins [11,12]. Very little Nrf2 is found in unstimulated cells and that which is present is held in cytoplasm by the actin-bound protein Keap1, which also promotes the degradation of Nrf2 by the proteasome. Upon stimulation by agents, which activate the ARE, Nrf2 is released from Keap1, which leads to its accumulation and its translocation to the nucleus, where it can induce the expression of genes containing an ARE [11]. NAD(P)H: quinone oxidoreductase (NQO1) functions as an important part of cellular antioxidant defense by detoxifying quinones, thus, NQO1 plays an important role in maintaining the redox balance in cells [28]. Recent studies have clearly shown that HO-1 assumes a central role in cellular antioxidant defense and especially, in vascular protection [29]. Like other phase II genes, the enhancer regions of both GCSL and GCSH contain the ARE, which is most critical for basal and inducible expression for GCS [30,31]. The mechanisms of tanshinone IIA's vascular protection might be related with Nrf2-mediated expression of the antioxidant genes. Knock down of Nrf2 expression by siRNA of Nrf2 could counteract tanshinone IIA's up-regulation intracellular GSH levels.

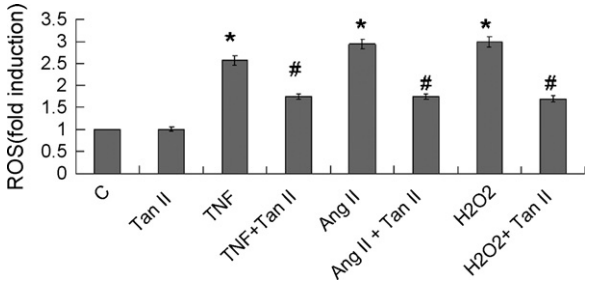


**Fig. 6 – Effects of tanshinone IIA on TNF-α, $H_2O_2$ or Ang II-induced intracellular ROS generation in HASMCs. HASMCs were treated with 5 μM tanshinone IIA for 30 min before treatment with TNF-α (10 ng/mL), $H_2O_2$ (100 μM) or Ang II (100 nM) for 15 min; and ROS were quantitatively analyzed. The percentage of ROS generation is indicated on the ordinate and related to the value for the control with no additives (the value for control is 84,960 ± 1326). $^{*}p < 0.05$ compared to the value of control, $^{\#}p < 0.05$ compared to the value with TNF-α, $H_2O_2$ or Ang II.**

Phosphorylation of the Nrf2/Keap1 complex, triggered by kinase signaling pathways, might be another possibility for the release of Nrf2 from the Nrf2/Keap1 complex. Mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) and PERK signaling pathways have been shown to be involved in Nrf2-mediated gene regulation [32–34]. The activation of extracellular signal regulated kinase (ERK) was responsible for the nuclear translocation of Nrf2 during the pyrrolidine dithiocarbamate induction of GCLM gene expression in HepG2 cells [35]. By using chemical inhibitors to investigate signaling pathways, PI3K was shown to be involved in the activation of Nrf2-mediated gene expression triggered by antioxidants such as tBHQ or by oxidative stress, and treatment with PI3K inhibitors, such as LY294002 or wortmannin,

significantly decreased the nuclear translocation of Nrf2 [36,37]. Green tea polyphenol extracts stimulated the transcription of phase II enzymes by ARE activation probably utilizing the MAPK signaling pathway [38]. Resveratrol, considered as an active cardioprotective component of red wine, enhanced the activity of phase II enzymes [39]. It demonstrated that resveratrol up-regulates phase II antioxidant/detoxifying enzymes through Nrf2 activation, mediated by MAPKs or PKB/Akt [40]. Our results showed that activation of the PI3K and ERK, but not JNK or p38 signaling pathways by tanshinone IIA disrupted the Nrf2–Keap1 complex and increases the nuclear translocation of Nrf2.

Chemopreventive agents activate Nrf2-mediated gene expression either by directly modifying the cysteine residues on Keap1 to disrupt the Nrf2–Keap1 complex, or by activating kinase signaling pathways such as MAPKs, PKC, and PI3K to phosphorylate the Nrf2/Keap1 complex and facilitate the release of Nrf2, or to increase the nuclear translocation of Nrf2 and regulate the transcriptional activity of Nrf2 nuclear co-activators. Nrf2 induction of phase II enzymes contributes to cytoprotection and potentially to self-repair of cells exposed to oxidative stress.

Sodium tanshinone IIA sulfonate (STS) protect ischemia-reperfusion injury through an electron transfer reaction in mitochondria against forming reactive oxygen radicals [41]. Tanshinone IIA has also been shown to inhibit LDL oxidation [42] as well as ANG II activity, resulting in attenuation of cardiac cell hypertrophy [43]. Our results demonstrated that tanshinone IIA provided cytoprotective effect through inhibition of ROS (mainly $H_2O_2$) production.

In summary, tanshinone IIA activates Nrf2-mediated gene expression either by directly modifying the cysteine residues on Keap1 to disrupt the Nrf2–Keap1 complex, or by activating kinase signaling pathways such as ERK and PI3K to phosphorylate the Nrf2/Keap1 complex and facilitate the release of Nrf2, or to increase the nuclear translocation of Nrf2 and regulate the transcriptional activity of Nrf2 nuclear co-activators. Therefore we propose that the magnitude of Nrf2 activation by tanshinone IIA in a pro-oxidative environment may be critical towards the protection against the damaging effects observed during oxidative stress. Determining whether tanshinone has in vivo efficacy would further be the potential therapeutic pharmacology of this compound.

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