Journal of Ethnopharmacology 145 (2013) 152–161

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Hydrogen peroxide-mediated oxidative stress and collagen synthesis in cardiac fibroblasts: Blockade by tanshinone IIA

Ping Wang [a,c,1], Sigui Zhou [b,c,1], Lipeng Xu [d,1], Yao Lu [e], Xi Yuan [e], Huijie Zhang [c], Ruifang Li [c], Jian Fang [c], Peiqing Liu [c,*]

[a] Shenzhen Institute for Drug Control, Shenzhen, China
[b] Department of Pharmacology, GuangDong Pharmaceutical University, Guangzhou, China
[c] Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China
[d] Institute of New Drug Research and Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Jinan University College of Pharmacy, Guangzhou, China
[e] Clinical Medicine Eight Years 1st Class 2007 Grade, Zhongshan School of Medicine, Sun Yat-sen University, GuangZhou, China

ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form
14 October 2012
Accepted 25 October 2012
Available online 16 November 2012

Keywords:
Hydrogen peroxide
Collagen synthesis
Oxidative stress
NAD(P)H oxidase
Tanshinone IIA

ABSTRACT

Ethnopharmacological relevance: We have recently reported that tanshinone IIA attenuated cardiac fibrosis in two-kidney, two-clip renovascular hypertensive rats via inhibiting NAD(P)H oxidase. However, little is known about the cellular and molecular mechanisms of tanshinone IIA mediated anti-fibrotic effects in cardiac fibroblasts after H_2O_2 stimulation. The present study was performed to investigate whether H_2O_2 may increase collagen synthesis in cardiac fibroblasts by affecting the expression and activity of NAD(P)H oxidase and whether the effects of H_2O_2 on cardiac fibroblasts can be blocked by treatment of tanshinone IIA.

Materials and methods: Cardiac fibroblasts were treated with H_2O_2 (100 μmol/L) in the presence or absence of tanshinone IIA (1 μmol/L), NAD(P)H oxidase inhibitors diphenyleneiodonium (10 μmol/L), siRNA-p47phox, siRNA-Nox2 and siRNA-Nox4. Collagen synthesis was measured by [^{3}H]proline incorporation, O_2^- production were determined by flow cytometry and DHE fluorescence microscopy. NAD(P)H oxidase activity was measured by lucigenin-enhanced chemiluminescence.

Results: H_2O_2 induced the activity of NAD(P)H oxidase, O_2^- production, collagen synthesis and fibronectin expression in cardiac fibroblasts, and DPI abolished this induction. Exposure of adult rat cardiac fibroblasts to H_2O_2 had time-dependent increase in the expression of p47phox, Nox2 and Nox4 oxidases. In addition, tanshinone IIA significantly inhibited H_2O_2-induced collagen synthesis via attenuation of O_2^- generation and NAD(P)H oxidase activity. Moreover, siRNA-mediated knockdown of p47phox, Nox2 and Nox4 inhibited H_2O_2-induced NADPH oxidase activity. H_2O_2-induced collagen synthesis and fibronectin expression were also inhibited by p47phox, Nox2 and Nox4 knock down.

Conclusions: Our data show that NAD(P)H oxidase plays a significant role in regulating collagen synthesis in H_2O_2-stimulated cardiac fibroblasts. Inhibition of NAD(P)H oxidase with tanshinone IIA completely blocked the H_2O_2-stimulated collagen production, which will raise the experimental basis for using tanshinone IIA to cardiac fibrosis in clinic.

1. Introduction

Cardiac fibrosis is one of the most important factors contributing to the transition from compensated cardiac hypertrophy to heart failure, which defined as interstitial fibroblast proliferation and excessive extracellular matrix deposition, leads to heart failure, sudden cardiac death and other serious complications (Krenning et al., 2010). Important components of cardiac extracellular matrix include structural and adhesive proteins such as collagen and fibronectin. Cardiac fibroblasts are the major source of collagen in the myocardium, which represent the most numerous cell type in the mammalian heart (D'Souza et al., 2011; Dixon and Cunnington, 2011). Therefore, inhibiting the process of cardiac fibrosis and collagen synthesis is a crucial strategy in clinical treatment.

Oxidative stress, a chronic increase in reactive oxygen species, can contribute to cardiac remodeling (Zhu et al., 2006; Takimoto

Abbreviations: Tan IIA, Tanshinone IIA; DPI, diphenyleneiodonium; DHE, dihydroethidium; H_2O_2, Hydrogen peroxide; O_2^-, superoxide anion; NAD(P)H, reduced form of nicotinamide-adenine dinucleotide (phosphate)

* Corresponding author. Tel.: +86 20 39943116; fax: +86 20 39943026.
E-mail address: liupqing1@163.com (P. Liu).

[1] Authors P.W., S.Z., and L.X. contributed equally to this work.

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http://dx.doi.org/10.1016/j.jep.2012.10.044

and Kass, 2007). The elevation of collagen production by oxidants and its amelioration by antioxidants strongly support a role of reactive oxygen species in cardiac fibrosis(Kai et al., 2006; Zhou et al., 2010; Worou et al., 2011). Recent work suggests a role for NAD(P)H oxidase in the development of hypertension, cardiac hypertrophy and interstitial fibrosis(Iwai et al., 2006; Barnes and Gorin, 2011). NAD(P)H oxidase is the major enzymatic sources of reactive oxygen species in the cardiovascular system and the only enzyme discussed so far whose primary function appears to be reactive oxygen species production (Muller and Morawietz, 2009; Xu et al., 2012). NAD(P)H oxidase is composed of a catalytic Nox2 subunit and a p22phox subunit and several cytosolic subunits (p47phox, p40phox, p67phox and Rac) that associate with the heterodimer in the activated enzyme. Five Nox isoforms (Nox1-5) form the basis of distinct NAD(P)H oxidase(Trott et al., 2011; Mozaffari et al., 2011). Therefore, therapeutic strategies aimed at alleviating or preventing reactive oxygen species injuries and NAD(P)H oxidase might be a reasonable choice for the treatment of cardiac fibrosis.

It has been shown that H_2O_2 induced oxidative stress, but a precise mechanism of the mediation of H_2O_2-induced oxidative stress remains unclear. The purpose of this study was to investigate whether H_2O_2 may increase collagen synthesis in cardiac fibroblasts by oxidative stress. Furthermore, we sought to identify the primary source of stimulated reactive oxygen species. Our results demonstrate that H_2O_2-stimulated collagen generation is related with the activation of NAD(P)H oxidase in cardiac fibroblasts, and which is highly sensitive to diphenyleneiodonium (DPI).

Tanshinone IIA (Tan IIA) is a major component of *Salvia miltiorrhiza* Bunge known as Danshen which has long been used for prevention and treatment of cardiovascular diseases in China (Ren et al., 2010; Zhao et al., 2011; Gao et al., 2012). Accumulating studies show that Tan IIA possesses many biological properties, largely depending on its anti-oxidative effects (Yang et al., 2008; Chen et al., 2012). Previous studies from our laboratory have found that Tan IIA can inhibit atherosclerotic calcification mediated by decreasing the oxidation of LDL and protect cardiac myocytes against apoptosis due to its antioxidant properties (Fu et al., 2007; Tang et al., 2007). Recently, we have reported that Tan IIA attenuated cardiac fibrosis in two-kidney, two-clip (2K2C) renovascular hypertensive rats by inhibiting NAD(P)H oxidase (Wang et al., 2011). However, it is not known if Tan IIA can inhibit collagen production in cardiac fibroblasts after H_2O_2 stimulation. Particularly, it is not known whether Tan IIA affects the NAD(P)H oxidase system in adult rat cardiac fibroblasts. Therefore the present study aimed to investigate the effects and mechanisms of Tan IIA on collagen production in cardiac fibroblasts induced by H_2O_2.

2. Materials and methods

2.1. Materials

Tan IIA was provided by professor Gu Lianquan (Institute of Pharmacy Synthesis, Sun Yat-sen University) and its purity is over 98% (assayed by HPLC). The structure of Tan IIA is shown in Fig. 1. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum were obtained from Life Technologies, Inc. H_2O_2 was obtained from Calbiochem, Lucigenin, NADH, NADPH, diphenyleneiodonium (DPI) were purchased from Sigma. CM-H2DCFDA and dihydroethidium (DHE) were from Invitrogen. [^{3}H]-thymidine and [^{3}H]-proline was obtained from Beijing Atom High Tech Co. Ltd. Monoclonal anti-α-tubulin were obtained from Sigma Aldrich. Polyclonal anti-Nox2 was obtained from BD, Inc. Polyclonal anti-Nox4, p47phox and fibronectin were obtained from Santa Cruz Biotechnology. siRNA-p47phox (sc-45918), siRNA-Nox2 (sc-61838), siRNA-Nox4 (sc-61887), control siRNA duplex (sc-37007) and siRNA tranfection reagent (sc-29528) were obtained from Santa Cruz Biotechnology.

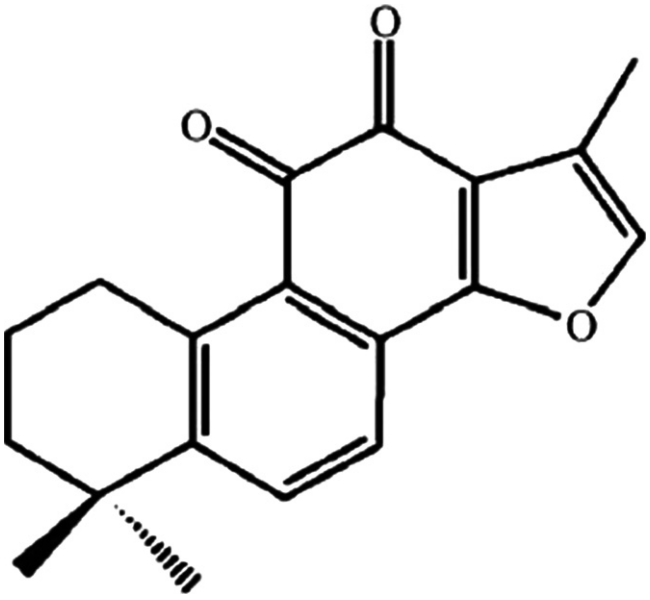

Fig. 1. The chemical structure of Tan IIA.

2.2. Culture of cardiac fibroblasts

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experiments were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University.

Primary cultures of adult rat cardiac fibroblasts were prepared as previously described (Fan et al., 2011). Briefly, male Sprague–Dawley rats (200–250 g) were anaesthetized using sodium pentobarbital (30 mg/kg, i.p.), after induction of deep anesthesia, the ventricles were isolated, minced, and digested in 0.25% collagenase solution (37 °C, 1 h). After digestion, the cells were pelleted and suspended in DMEM supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (FBS). The suspension was transferred to 56.7 cm^2 dishes. After a 60 min incubation period, cells that were weakly attached or unattached were removed, and the attached cells were grown to confluence. After 2–3 days, the confluent cells were detached by trypsin and seeded on new dishes. The purity of these cultured cardiac fibroblasts was greater than 95% on the basis of positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. Third to fifth passage cardiac fibroblasts were used for all experiments. Serum-containing medium from the cultured cells was replaced with serum-free medium, and the cells were then exposed to the agents as indicated.

2.3. Analysis of DNA and collagen synthesis

The effects of H_2O_2 on DNA and collagen synthesis in cardiac fibroblasts were evaluated by the incorporation of [^{3}H]-thymidine and [^{3}H]-proline into cells, respectively, as described previously (Hattori et al., 2006). To examine DNA synthesis, 0.5 μCi of [^{3}H]-thymidine was added 12 h after treatment with H_2O_2, and the cells were incubated for 12 h. To examine collagen synthesis, 0.5 μCi of [^{3}H]-proline was added 12 h after treatment with H_2O_2, and the cells were incubated for 24 h. After labeling, the cells were rinsed twice with cold PBS and incubated with 10% trichloroacetic acid at