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Preliminary report

Tanshinone IIA protects against subclinical lipopolysaccharide induced cardiac fibrosis in mice through inhibition of NADPH oxidase

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ABSTRACT

Myocardial fibrosis plays a central role in the development of heart failure. It has been shown that recurrent exposure to subclinical lipopolysaccharide (LPS) increases mortality and induces cardiac fibrosis in mice, which is not mediated by the common renin-angiotensin system. LPS increased NADPH oxidase2 (NOX2) in isolated adult mouse cardiac fibroblasts and NOX2 may mediate LPS-induced cardiac fibrosis. Therefore, the current study was designed to delineate the role of NOX2 in LPS-induced fibrosis model and to investigate the preventive role of Tanshinone IIA (TIIA) on the development of cardiac fibrosis. The protective mechanism of TIIA was determined to be associated with the inhibition of NOX2, by comparing its effects with the NADPH oxidase inhibitor, apocynin. The results revealed remarkable effects of apocynin and TIIA on attenuating the development of myocardial fibrosis and fibrosis-related genes and mediators. Furthermore, TIIA and apocynin decreased the expression of NADPH oxidase subunits (NOX2 and $P67^{phox}$) expression and the ROS levels. The anti-fibrotic effect of apocynin suggested that NOX2 inhibition may be a potential preventive strategy for attenuating the progression of LPS-induced cardiac fibrosis. Our results demonstrate that TIIA may be a potent agent against subclinical LPS-induced cardiac fibrosis in mice partially via inhibition of NADPH oxidase 2.

1. Introduction

The cardiac extracellular matrix (ECM) is a dynamic structure, which can adapt to various physiological and pathological stresses placed on the heart [1]. Myocardial fibrosis (MF) is characterized by net accumulation of extracellular matrix proteins in the cardiac interstitium and results in both systolic and diastolic dysfunctions [2]. Cardiac fibrosis is the common feature of advanced coronary heart disease, hypertension and cardiomyopathy, which results in an increased risk of morbidity and mortality [3,4]. Circulating subclinical lipopolysaccharide (LPS) also increases mortality and induces cardiac fibrosis, which occurs commonly and is seemingly well tolerated [5,6]. Exposure to LPS from gut microbiota induces a metabolic endotoxemia that may contribute to obesity, glucose intolerance, and insulin resistance [7]. These adverse effects develop insidiously with no change in activity, appetite, weight, blood chemistries, left ventricular size or systolic function [5].

Common mediators of cardiac fibrosis in pathological remodeling including the renin-angiotensin system (RAS), transforming growth factor-β (TGF-β) and TNF-α, do not appear to be involved in cardiac fibrosis with subclinical LPS [8]. There is some evidence that LPS increased NADPH oxidase2 (NOX2) in isolated adult mouse cardiac fibroblasts and NOX2 may mediate LPS-induced cardiac fibrosis [6]. The NOX family proteins are enzymes that are dedicated themselves to producing O_2^- and/or H_2O_2, a property which distinguishes them from other enzymes producing ROS as a byproduct [9,10]. NOX-derived ROS have been previously related to fibrosis in the heart [11]. Recent study has demonstrated a potential link between AngII-associated fibrotic response and NOX in cardiac fibroblasts [12]. Moreover, the role of NOX2 in LPS-induced cardiac fibrosis is not fully elucidated. Apocynin, a NOX inhibitor, protects against the LPS-induced acute lung injury in rats through its antioxidant and antiinflammatory effect [13].

Tanshinone IIA (TIIA) is one of the main components isolated from Danshen, which is widely used for the treatment of cardiovascular diseases [14]. In our previous study, TIIA attenuates cardiac dysfunction via reducing inflammatory cytokines release and inhibiting the NOX2 signaling during endotoxemia [15]. TIIA prevents cardiac remodeling through attenuating NADPH oxidase-derived reactive oxygen species (ROS) production in hypertensive rats [16]. However, there are few reports that examine whether TIIA also has a protective effect on myocardial fibrosis during LPS exposure. In the present study, the protective mechanism of TIIA was determined to be associated with the inhibition of NADPH oxidase, by comparing its effects with the positive control apocynin.

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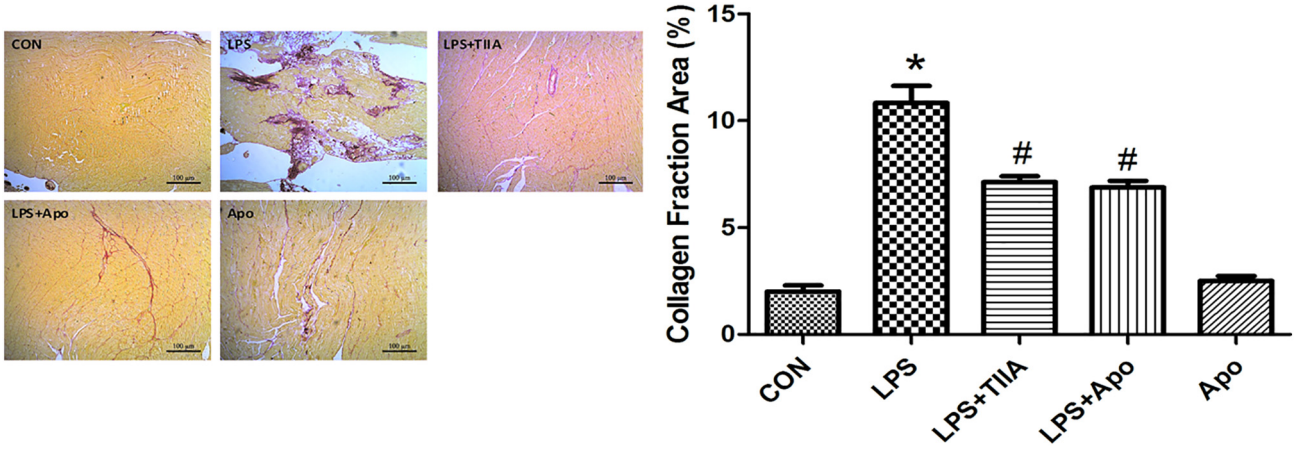

Fig. 1. T IIA and apocynin inhibited subclinical LPS induced cardiac fibrosis. Data are mean ± SEM. *$P < 0.05$, compared with the control group; #$P < 0.05$ compared with LPS group. n = 3.

2. Materials and methods

2.1. Experimental protocol

All animal care and experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, revised 2011) and the institutional guidelines of the Animal Care and Use Committee of Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. Male C57BL/6 mice weighing 25–30 g were randomized into five groups: control group (CON), LPS group, LPS+ TIIA group (TIIA 10 mg/kg intraperitoneal injection, once a day for 2 weeks), LPS + Apocynin group, and Apocynin group. Mice were injected i.p. with LPS at a dose of 10 mg/kg once a week for 2 weeks to induce fibrosis model. Mice were injected with 0.5 ml of saline as a control. Apocynin 30 mg/kg was given orally once daily for the entire experimental course [17].

2.2. Measurement of cardiac fibrosis

The samples from treatments were sectioned to a thickness of 5 μm. Percentage of fibrosis was determined with picrosirius red-stained tissue sections using Image Tool software (version3.0). Fibrosis area was expressed as the percentage of the area of total left-ventricular (LV) [6].

2.3. RT-PCR

Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was used to measure expression of fibrosis and hypertrophy related genes. Total RNA was extracted from LV samples, digested with RNase-free DNase, and reverse transcribed. QRT-PCR was performed and RNA equivalents normalized to simultaneously determine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample.

2.4. Western blot analysis

Ventricular tissues were collected for immunoblotting analysis as described in our previous studies. Briefly, cellular proteins were prepared, separated on 10% SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore). The membranes were probed with appropriate primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies. The signals were detected by enhanced Pierce chemiluminescence. The blots against β-actin served as loading controls. The signals were quantified by scanning densitometry and the results from each experimental group were expressed as relative integrated intensity compared with that of controls.

2.5. In situ detection of reactive oxygen species (ROS)

To evaluate heart ROS production in situ, frozen, unfixed, whole heart cross-sections were stained with 10 μmol/L DHE (Sigma) for 30 min in a dark humidified chamber at 37 °C. ROS generation was indicated by red fluorescence and visualized with fluorescence microscopy. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in fluorescence were expressed as an arbitrary unit.

2.6. Statistical analysis

Results are presented as mean ± SEM. The significance of the difference was performed using one-way analysis of variance (ANOVA) with Tukey's procedure for post-hoc comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. TIIA supplement inhibited subclinical LPS-induced cardiac fibrosis

To assess fibrosis, myocardial tissues were stained with picrosirius red to highlight collagen. Ratios of collagen area to total area were measured. Compared with control and apocynin group, LPS significantly increased collagen fraction area. Treatment with TIIA or apocynin partly abolished the LPS-induced increase in collagen fraction area ($P < 0.05$). However, there were no significant differences between the CON and Apo group (Fig. 1).

3.2. TIIA attenuated fibrotic response in the myocardium

To investigate whether LPS exposure stimulates the signaling cascade leading to cardiac fibrosis, we investigated mRNA levels of collagen Iα1, collagen IIIα1, MMP2, MMP9, TIMP1, TIMP2 in the heart. As shown in Fig. 2, LPS increased cardiac expression of several fibrosis-related genes 2 weeks after LPS exposure. TIIA and Apocynin cotreatment for 14 days significantly attenuated LPS-induced collagen Iα1, collagen IIIα1, MMP2, MMP9, TIMP1, TIMP2 mRNA expression in the heart ($P < 0.05$).