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Preventive but nontherapeutic effect of danshensu on hypoxic pulmonary hypertension

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Abstract

Objectives: Danshensu is a traditional Chinese medicine that is used for treatment of cardiovascular diseases. We previously demonstrated its preventive effect against early-stage hypoxic pulmonary hypertension (HPH) in a rat model. To determine whether danshensu treatment might be useful for patients with chronic HPH, we examined its therapeutic effect in rats with prolonged HPH.

Methods: Adult Sprague-Dawley rats received danshensu (80, 160, and 320 mg/kg) during or after hypoxia exposure to assess preventive and therapeutic effects, respectively. Right ventricle systolic pressure (RVSP), right ventricle hypertrophy index (RVHI), and mean left carotid artery pressure (mCAP) were measured in each group. Western blotting was used to assess transforming growth factor (TGF)-β expression levels in rats and cultured cells exposed to hypoxia.

Results: Preventive danshensu treatment significantly reduced the elevation of RVSP and RVHI in rats exposed to hypoxia, whereas therapeutic danshensu treatment did not; mCAP did not change in any treatment group. The increased expression levels of TGF-β induced by hypoxia were inhibited by preventive danshensu treatment, but not by therapeutic danshensu treatment.

Conclusions: Although danshensu treatment could prevent HPH, it had no obvious therapeutic effect after development of HPH. Therefore, danshensu might be suitable for clinical treatment of early-stage HPH.

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Danshensu, hypoxic pulmonary hypertension, preventive treatment, therapeutic treatment, TGF-β, 3,4-dihydroxyphenyllactic acid, Chinese traditional medicine, blood pressure, hypertrophy

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Introduction

Hypoxic pulmonary hypertension (HPH) is a common disorder caused by situations involving restricted oxygen supply, such as chronic obstructive pulmonary disease, cor pulmonale, and high altitude pulmonary hypertension.[1–3] In this disorder, pathophysiological processes involve endothelial dysfunction and proliferative vascular remodeling of the pulmonary arteries; these changes might eventually lead to right ventricular heart failure and death.[4–6] However, there are currently no effective cures for this devastating disease.

We previously reported that danshensu could prevent HPH in rats.[7] Danshensu is a major active component of *Salvia miltiorrhiza*; this Chinese traditional medicine that has been widely used in China and many other countries.[8,9] Danshensu functions by dilating cardiac arteries, inhibiting thromboxane formation, reducing platelet adhesion and aggregation, and scavenging free radicals.[10–12] In our previous study, we used a rat model with early-stage HPH; thus, it remains unclear whether danshensu is beneficial for patients with chronic HPH.[7] Here, we examined the therapeutic effect of danshensu in rats with prolonged HPH. This analysis might provide insights regarding the usefulness of danshensu for patients with chronic HPH.

Materials and methods

Chemical reagent

Danshensu (purity, 99.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); it was then dissolved in distilled water to yield a stock solution of 5 mg/mL.

Animal experiments

All experiments were approved by the Animal Care and Use Committee of the Fourth Affiliated Hospital of Hebei Medical University; they were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The experimental design was consistent with the recently published guidelines for preclinical research in pulmonary hypertension.[13]

Sprague-Dawley rats (180–220 g) were randomly divided into five groups (n = 6 per group, three males and three females): normoxia (N), hypoxia (H), hypoxia with 80 mg/kg danshensu treatment (H + DSS80), hypoxia with 160 mg/kg danshensu treatment (H + DSS160), and hypoxia with 320 mg/kg danshensu treatment (H + DSS320). N group rats were maintained on room air, while H group rats were maintained in a specially designed hypobaric chamber depressurized to 380 mmHg (oxygen concentration reduced to approximately 10%) for 8 hours daily.

For the preventive study, danshensu was intraperitoneally injected daily into H group rats at doses of 80, 160, and 320 mg/kg, beginning when the rats underwent exposure to hypoxia and continuing for 4 weeks. For the therapeutic study, danshensu was intraperitoneally injected at the same doses for 2 weeks after rats had been exposed to hypoxia. Equivalent volumes of

saline were injected into rats in the N and H control groups.

The rats were anesthetized via peritoneal injection of pentobarbital (≥30 mg/kg) for the measurement of selected parameters. The right ventricle systolic pressure (RVSP) was measured by a polyethylene micro-catheter inserted from the right external jugular vein into the right cardiac ventricle. The mean carotid artery pressure (mCAP) was measured by a polyethylene micro-catheter inserted from the left common carotid artery. The anesthetized rats were then euthanized by exsanguination and their hearts were collected for analysis. The right ventricle (RV) and the left ventricle (LV) with septum (S) were isolated and individually weighed to derive the right ventricle hypertrophy index (RVHI): RV/(LV+S).

Cell culture and treatment

After rats had been euthanized (described in the previous section), pulmonary arteries were rapidly isolated for collection of pulmonary artery fibroblasts (PAFs). The harvested cells were either flash frozen at −80°C for subsequent western blotting or cultured (using RPMI 1640 medium with 20% fetal bovine serum [Gibco, Grand Island, NY, USA]) in 5% CO_2 and 95% air at 37°C. Experimental treatments were performed after three to five generations of subculture. PAFs were identified by vimentin staining (>90% of cells were positive). O_2 was used at concentrations of 21% and 3% for normoxia and hypoxia treatments, respectively.

Western blotting

Cultured cells or isolated tissues were lysed in a solution of 8 M urea and 2% sodium dodecyl sulfate with 1x protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were measured by the bicinchoninic acid method (Thermo Fisher Scientific, Waltham, MA, USA), and 20 μg protein from each sample was used for western blotting. Individual samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4%–12% gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 15 minutes at 20°C with 5% dry milk in Tris-buffered saline with Tween 20 (TBST), then incubated with anti-TGF-β1 (Abcam, Cambridge, MA, USA; Cat. No. ab179695; 1:1000 dilution in 3% bovine serum albumin in TBST) and anti-β-actin (Abcam; Cat. No. ab8226; 1:1000 dilution in 3% bovine serum albumin in TBST) antibodies for 1 hour at 20°C. Membranes were washed in TBST, then incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam; Cat. No. ab6721 [for anti-TGF-β1 primary antibody] and ab6728 [for anti-β-actin primary antibody]; 1:10000 dilution in 3% bovine serum albumin in TBST) for 30 minutes at 20°C. Antibody-protein reactions were visualized using an enhanced chemiluminescence reagent (Bio-Rad, Hercules, CA, USA). Quantitation of bands was performed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Briefly, relative signal densities of bands and surrounding background were measured and subtracted for each sample. The net signal density for each sample was then normalized to the average signal density for all control samples; this ratio was recorded as the relative expression level for each sample.

Statistical analyses

All data are presented as mean ± standard deviation. One-way analysis of variance was used to assess statistical differences; pairwise analysis was performed by post hoc Tukey tests. All statistical analyses

were performed using SPSS Statistics, version 25.0 (IBM Corp., Armonk, NY, USA). Differences with $P < 0.05$ were considered statistically significant.

Results

Preventive effect of danshensu on HPH

In the preventive experiment, the rats received danshensu during exposure to hypoxia. The RVSP, a parameter that reflects pulmonary artery pressure, increased from 25.19 ± 4.13 mmHg in the N group to 48.17 ± 6.62 mmHg in the H group ($P < 0.05$ relative to the N group). However, pretreatment with danshensu at 80, 160, and 320 mg/kg reduced the RVSP values to 44.31 ± 5.24 mmHg, 35.31 ± 7.13 mmHg, and 31.16 ± 5.96 mmHg, respectively (all $P < 0.05$ relative to the H group; Figure 1a).

In addition, ventricle hypertrophy was examined by the RVHI, which assessed ventricular muscle weight. The RVHI values were 0.21 and 0.39 in the N and H groups ($P < 0.05$); they were 0.37, 0.29, and 0.25 when rats exposed to hypoxia received danshensu treatments at 80, 160, and 320 mg/kg, respectively (all $P < 0.05$ relative to the H group; Figure 1b). As expected, hypoxia and danshensu treatment did not affect the mCAP, which was mainly determined by the left ventricular pressure (Figure 1c). These results confirmed the preventive effect of danshensu on HPH in the rat model.

Therapeutic effect of danshensu on HPH

In the therapeutic experiment, danshensu was administered after 2 weeks of hypoxia exposure. The RVSP was 24.39 mmHg in the N group, whereas it was 51.73 mmHg in the H group ($P < 0.05$). This elevation was not alleviated by danshensu treatment; rats exposed to hypoxia showed RVSP values of 48.63, 46.25, and 45.13 mmHg at danshensu doses of 80, 160, and 320 mg/kg, respectively (Figure 2a).

Furthermore, RVHI values were 0.20 and 0.43 in the N and H groups ($P < 0.05$); they were 0.39, 0.38, and 0.37 when rats exposed to hypoxia received danshensu treatments at 80, 160, and 320 mg/kg, respectively

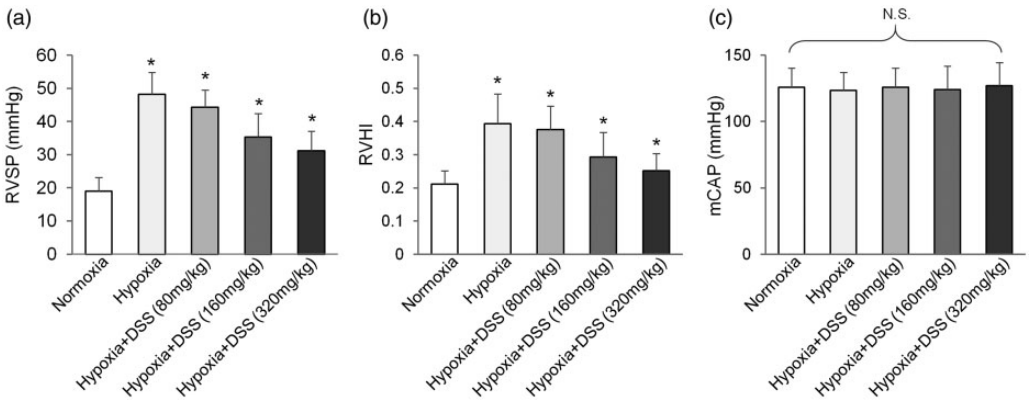

Figure 1. Preventive effects of danshensu in rats exposed to hypoxia. Measurements of (a) RVSP (hypoxia group compared with normoxia group; all others compared with hypoxia group), (b) RVHI (hypoxia group compared with normoxia group; all others compared with hypoxia group), and (c) mCAP. In this experiment, DSS was administered concurrently with hypoxia induction. *, $P < 0.05$; N.S., not statistically significant; DSS, danshensu; RVSP, right ventricle systolic pressure; RVHI, right ventricle hypertrophy index; mCAP, mean carotid artery pressure.

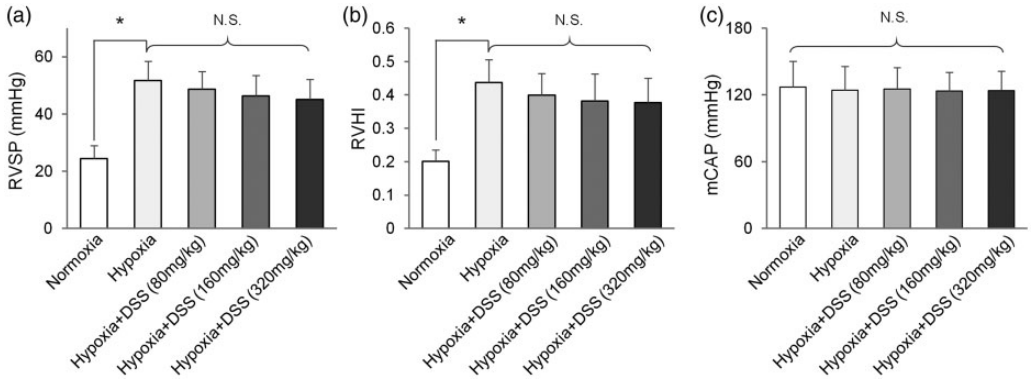

Figure 2. Therapeutic effects of danshensu in rats exposed to hypoxia. Measurements of (a) RVSP, (b) RVHI, and (c) mCAP. In this experiment, DSS was administered after rats had been exposed to hypoxia for 2 weeks. *, $P < 0.05$; N.S., not statistically significant; DSS, danshensu; RVSP, right ventricle systolic pressure; RVHI, right ventricle hypertrophy index; mCAP, mean carotid artery pressure.

(Figure 2b). Pairwise comparisons revealed no significant differences between the H group and the danshensu treatment groups. In addition, no significant differences in mCAP were observed among the five groups of rats (Figure 2c).

Effect of danshensu on TGF-β expression during preventive and therapeutic treatment

In this study, danshensu treatment was preventive against HPH, but not therapeutic for existing HPH. TGF-β has been widely reported to play an important role in the response to hypoxia[14–17] and we previously demonstrated that it is involved in the protective effect of danshensu against HPH.[7] Therefore, we sought to determine whether the differences in effects of danshensu were related to the regulation of TGF-β expression. In PAFs isolated from normal rats, administration of danshensu concurrently with exposure to hypoxia suppressed the expression of TGF-β, as expected ($P < 0.05$ relative to the H group); however, danshensu failed to exert this suppressive effect when it was added after the cells had been exposed to hypoxia for 24 hours (Figure 3a). These results suggested that danshensu inhibited the elevation of TGF-β during hypoxia, but could not return TGF-β expression levels to normal after exposure to hypoxia. To confirm this finding, we performed western blotting of TGF-β using frozen PAFs from the experimental rats in this study. Indeed, the expression of TGF-β in rats could be inhibited by danshensu during exposure to hypoxia ($P < 0.05$ relative to the N group), but could not be returned to normal after exposure to hypoxia (Figure 3b).

Discussion

In this study, we found that the therapeutic effect of danshensu on prolonged HPH was limited, which might be related to its inability to control TGF-β expression after exposure to hypoxia. We previously demonstrated that danshensu could prevent HPH in rats by inhibiting the proliferation of pulmonary artery smooth muscle cells through the TGF-β pathway.[7] However, the present study showed that once TGF-β is induced, its expression level could not be returned to normal by danshensu treatment; this

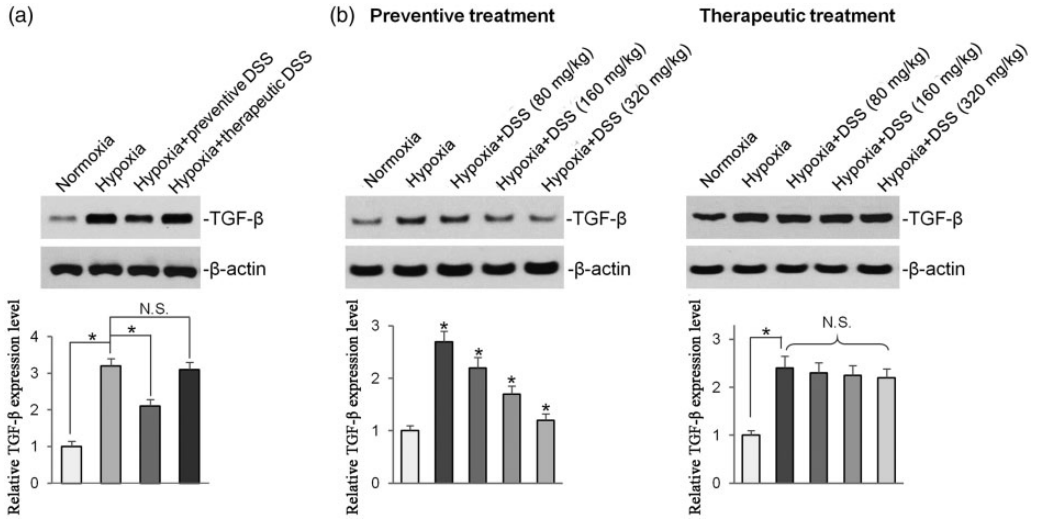

Figure 3. Effects of danshensu on expression of TGF-β during exposure to hypoxia. (a) Expression of TGF-β in PAFs isolated from normal rats in response to hypoxia and DSS treatments. DSS (30 μg/mL) was administered concurrently with hypoxia induction (Preventive) or after exposure to hypoxia for 24 hours (Therapeutic). Three independent experiments were used for statistical analysis. (b) PAFs isolated from experimental rats in Figure 1 and Figure 2 were prepared for western blotting of TGF-β (hypoxia group compared with normoxia group; all others compared with hypoxia group). *, $P < 0.05$; N.S., not statistically significant; DSS, danshensu; PAFs, pulmonary adventitial fibroblasts; TGF, transforming growth factor.

finding suggested that danshensu targets the upstream portion of the TGF-β pathway.

The expression of TGF-β could be induced by exposure to hypoxia, potentially through inhibition or transcriptional activation of hypoxia-inducible factor-1 (HIF-1).[18–20] It will be useful to determine whether danshensu suppressed the expression of TGF-β alone or whether it also suppressed other cytokines downstream of HIF-1. The underlying mechanism might be addressed by examination of the HIF-1 protein expression level and the mRNA levels of its target cytokines during danshensu treatment in rats with HPH.

Notably, the current study demonstrated that the therapeutic effect of danshensu was limited in rats and cells exposed to hypoxia; moreover, danshensu was unable to return the expression levels of TGF-β to normal after exposure to hypoxia. With respect to clinical treatment, we propose that danshensu should be used as early as possible for patients with early-stage HPH, and that it might be particularly useful for patients with acute HPH.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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