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RESEARCH ARTICLE

Influence of Salidroside on Expression Level of Endothelin-1 and Its Receptors under Hypoxic Conditions in Chicken Embryonic Pulmonary Artery Smooth Muscle Cells

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ABSTRACT

Salidroside (SDS), a traditional Chinese medicine, possesses many important functional activities such as anti-hypoxia, anti-fatigue, enhancing work performance, and is especially famous in the treatment of mountain mal-hypoxia in Tibet. Here we designed the study to evaluate the pharmacological effect of SDS on expression of ET-1 and its receptors in hypoxia-treated pulmonary artery smooth muscle cells (PASMCs). Chicken embryonic PASMCs incubated with or without SDS for 1h were then subjected to the hypoxic exposure for 24h. We evaluated the expression levels of ET-1 and its receptors mRNA in treated PASMCs. Compared with the hypoxia-treated group, the levels of ET-1 gene expression were notably decreased in both the moderate dose group (80μg/ml) and the high dose group (120μg/ml) ($P<0.01$, $P<0.05$) respectively, but no obvious decrease was found in the low dose group (40μg/ml). Meanwhile, pretreatment with SDS (40, 80, 120μg/ml) was found to be effective in reducing ($P<0.01$, $P<0.01$, $P<0.05$) the expression levels of ETA in avian PASMCs exposed to hypoxic conditions *in vitro*. Moreover, pretreatment with SDS markedly attenuated ($P<0.01$) ETB mRNA expression levels in the low-dose group (40μg/ml) and the moderate-dose group (80μg/ml), while in high dose group (120μg/ml), there was no obvious decrease. Collectively, our findings showed that SDS has a significant influence on the expression of ET-1 and its receptors in hypoxia-treated avian PASMCs.

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INTRODUCTION

Endothelin-1 (ET-1), a powerful endothelium-derived vasoconstrictor produced by vascular endothelial cells, and was reported to have various effects in the pulmonary vasculature (Gao *et al.,* 2012). This 21-amino-acid protein possibly involved in a number of serious vascular events, such as pulmonary hypertension (PH), a disease featured by abnormal smooth muscle cell growth and remodeling of the pulmonary vasculature (Giaid, 1998; Chen *et al.,* 2000; Stanford *et al.,* 2004; Wilson *et al.,* 2012; Mazurek *et al.,* 2013; Solcan *et al.,* 2015). Furthermore, PH followed by ascites has been recognized as a prominent cause of morbidity and mortality in commercial broiler production (Xi *et al.,* 2012; Gao *et al.,* 2013).

Salidroside (SDS), a traditional Tibetan medicine, was known as the main active ingredient in the root of *Rhodiola rosea* L. (Crassulaceae), and was suggested to have various pharmacological properties, including anti-hypoxia (Wang *et al.,* 2012), anti-fatigue and anti-stress activity (Zhang *et al.,* 2012), enhancing work performance and preventing high altitude sickness. It is especially famous in the treatment of mountain mal-hypoxia in China (Wu *et al,* 2008). Hence, SDS has been used in such special posts as astronauts, pilots, divers and mountaineers to enhance the ability for survival in adverse environment (Ming *et al.,* 1998). Recently, the protective effect of SDS has been elucidated in human derived cell lines attenuate H_2O_2-treated cell apoptosis (Zhang *et al.,* 2007). SDS has also been suggested to attenuate the amyloid-beta induced cytotoxicity in PC12 cells (Li *et al.,* 2010). However, the influence of SDS on expression of

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ET-1 and its receptors in chicken embryonic pulmonary artery smooth muscle cells (PASMCs) subjected to hypoxic conditions is still unclear. Here we designed the study to evaluate the pharmacological effect of SDS on expression of ET-1 and its receptors in hypoxia-treated PASMCs.

MATERIALS AND METHODS

Materials: SDS was obtained from Yuanye Technology Co. Ltd., Shanghai, China. All chemical reagents used in this study were of analytical grade.

Primary chicken embryonic PASMCs culture: Isolation and culture of avian PASMCs were done by a modification of the method of Li (Li *et al.*, 2009). All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University. Briefly, twenty 18-day-old embryonated broiler eggs were procured and perfused with PBS through the right ventricle. After flushing out the blood from the pulmonary circulation, the pulmonary artery was removed and washed in a petri dish with ice-cold PBS containing 100U/ml penicillin and 100μg/ml streptomycin. After stripping off the adventitia membrane, the pulmonary vascular intima was longitudinally cut and the endangium was scrapped with a blade to remove the endothelial cells. All pulmonary artery tissues were collected, minced finely and then resuspended in M199 containing 20% FBS and 1% antibiotic/antimycotic for 2min. The tissue suspension was centrifuged at 500g for 4min and the supernatant was discarded. The pulmonary tissue was aspirated into 25cm^2 tissue culture flasks using a sterile glass Pasteur pipette and then placed in a CO_2 incubator for 3h. M199 complete culture medium (containing 15% FBS, 90U/ml heparin and 1% antibiotic/antimycotic) was added to infiltrate the pulmonary tissue and transferred into a 5% CO_2 incubator for further expansion. On adding M199 complete culture medium after 24h, the tissue pellets were subsequently removed at 72h.

***In vitro* hypoxia procedure:** *In vitro* hypoxia model was achieved following an earlier procedure (Li *et al.*, 2013). The isolated PASMCs were grown to confluence and then were cultured in DMEM containing 10% FBS, 300μg/ml G418, 1% glutamine and 1% non-essential amino acids at 37ºC with 5% CO_2. Fourteen hours before SDS incubated, the original culture media was replaced with FBS-free DMEM media. Before hypoxia challenge, avian PASMCs were treated with various doses (40, 80, 120μg/ml) of SDS for 1h then cultured in a 37ºC chamber containing 1% O_2 and 5% CO_2 for another 24h. PASMCs cultured under normoxic conditions as a control.

PASMCs viability determination: Avian PASMCs viability was measured by MTT assay and the results are presented in Fig. 1.

RNA Isolation and cDNA Synthesis: Total RNA from the harvested avian PASMCs was isolated according to the acid phenol-chloroform procedure, and then that was reversed into cDNA using PrimeScript RT reagent Kit.

Quantitative real-time-PCR (qRT-PCR) to evaluate the gene expression of ET-1, ETA and ETB in PASMCs: qRT-PCR was conducted in quadruplex using the ABI StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sequences were as follows: GAPDH, sense, 5'- CCTTCATTGACC TTCACTACATGGTCTA-3', and antisense, 5'- TGGAA GATGGTGATGGCCTTTCCATTG-3'; ET-1, sense, 5'-GGACGAGGAGTGCGTGTATT-3', and antisense, 5'-GCTCCAGCA AGCATCTCTG-3'; ETA, sense, 5'-GTGGCCTTTTGGAGATTCTG-3', and antisense, 5'-GATTCCGATTCCCTGAACAC-3'; ETB, sense, 5'-CATCATCGACATCCCCATCA-3', and antisense, 5'-CACTAATTTACACATTTCGACACCAA - 3'.

Statistical analysis: All statistical analyzes were performed using SPSS 14.0 for Windows software (SPSS, Chicago, IL, USA). Quantitative variables are expressed as mean±SD. Differences in real-time PCR FC were determined using a Student's t test. $P<0.05$ was considered statistically significant.

RESULTS

SDS against hypoxia-induced cell damage: PASMCs viability data was presented in Fig. 1 employing the MTT method. Hypoxia for 24h resulted in markedly reduced in PASMCs survival. Compared to the normoxic group, only 48% viable PASMCs were observed. As illustrated in Fig. 1, SDS (40, 80, 120μg/ml) prevented avian PASMCs from hypoxia-induced loss, increasing cells survival to 56±2.73, 65±2.91 and 72±3.89, respectively. Furthermore, the beneficial influence of SDS was also be verified by the cellular image analysis (Fig. 2). After hypoxia treatment, avian PASMCs became oval and round in morphology and SDS pretreatment can be effective against these adverse effects.

SDS Decreases ET-1 Expression in hypoxia-induced PASMCs: The up-regulation of expression of ET-1 in chicken embryonic PASMCs *in vitro* was evaluated by treating under hypoxic conditions for 24h. qRT-PCR analysis showed 1.3-fold increased expression of ET-1 mRNA as compared with the normal group (Fig. 3). To ascertain the effect of SDS on ET-1 mRNA expression, SDS was applied onto hypoxia-induced PASMCs for 24h. As shown in Fig. 3, compared with the hypoxia-treated group, among the various concentrations of SDS pretreatment, the levels of ET-1 gene expression were significantly decreased ($P<0.01$, $P<0.05$, respectively) in both the moderate dose group (80μg/ml) and the high dose group (120μg/ml), but no obvious decrease was found in the low dose group (40μg/ml). The expression of ET-1 in PMVECs grown in SDS supplemental medium (80, 120μg/ml) for 24h was reduced to about 76 and 21%, respectively, as compared to only hypoxia-treated PASMCs. The largest effect was attributed to the group treated with 80μg/ml of SDS. These results strongly suggested that hypoxia could increase ET-1 expression and SDS preincubation significantly reduced hypoxia-induced excessively expression of ET-1 mRNA level in chicken embryonic PASMCs.

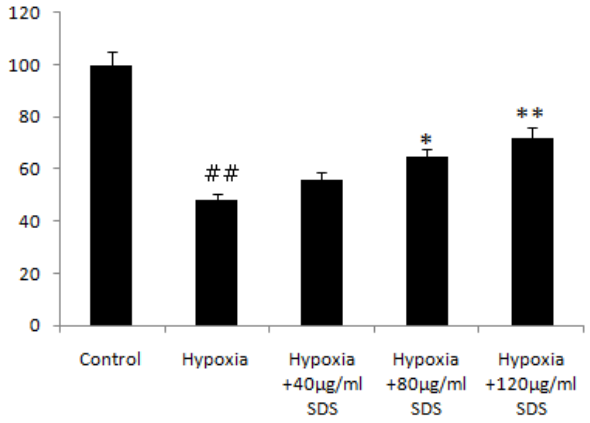

Fig. 1: Influence of SDS on hypoxia-treated cell survival in avian PASMCs. All data are showed as mean ± SD of eight wells in at least three independent experiments. # P<0.05, ## P<0.01 versus normal group; *P<0.05, **P<0.01 versus hypoxia-treated group

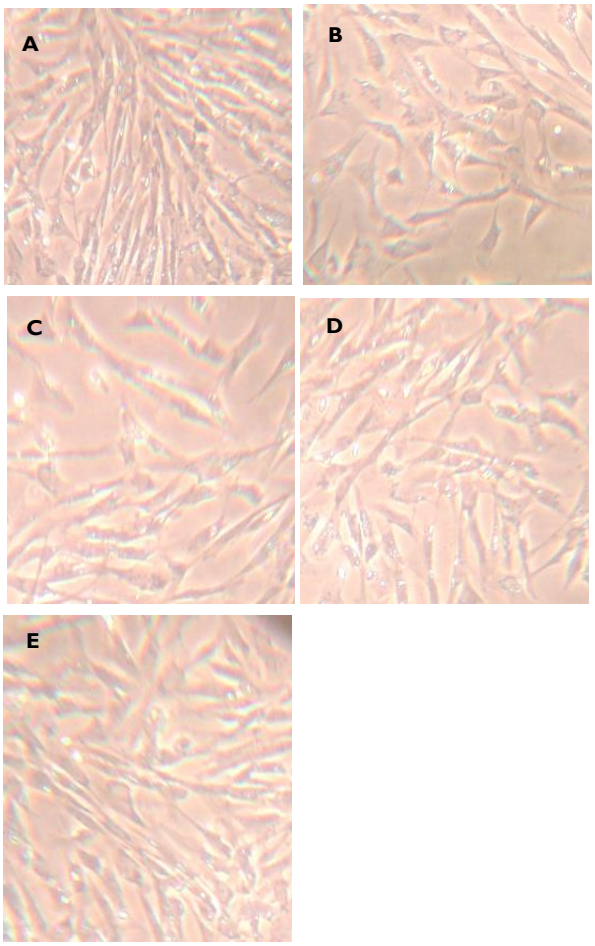

Fig. 2: Influence of SDS on hypoxia-treated shape changes in avian PASMCs (×200). A: Normal PASMCs; B: hypoxia PASMCs; C: hypoxia+40μg/ml SDS PASMCs; D: hypoxia+80μg/ml SDS PASMCs; E: hypoxia+120μg/ml SDS PASMCs.

SDS Decreases ETA Expression in hypoxia-induced PASMCs: We next investigated the influence of SDS pretreatment on the expression level of ETA mRNA in chicken embryonic PASMCs. As shown in Fig. 4, compared with the hypoxia-treated group, the levels of ETA gene expression were noticeable reduced (P<0.01, P<0.01, P<0.05, respectively) in all the SDS pretreatment group, after the PASMCs being treated with different SDS doses (40, 80, 120μg/ml), and the greatest effect was observed in the group treated with 40μg/ml of SDS. The expression of ETA in PASMCs grown in SDS supplemental medium (40, 80, 120μg/ml) for 24h were reduced to about 91, 89 and 32%, respectively, as compared to only hypoxia-treated PASMCs. Our findings suggested that SDS pretreatment could markedly reduce ETA gene expression in hypoxia-treated PASMCs.

SDS Decreases ETB Expression in hypoxia-induced PASMCs: We further tested the influence of SDS on the expression of ETB mRNA in chicken embryonic PASMCs exposed to hypoxic condition. As shown in Fig. 5, compared with the hypoxia-treated group, the levels of ETB mRNA expression were significantly decreased (P<0.01, P<0.01, respectively) in both the low-dose group (40μg/ml) and the moderate-dose group (80μg/ml), but there was no significantly decrease in the high dose group (120μg/ml). The expression of ETB in PASMCs grown in SDS supplemental medium (40, 80μg/ml) for 24h were reduced to about 46 and 52%, respectively, as compared to only hypoxia-treated PASMCs. These findings demonstrated that SDS pretreatment also has an influence on the expression of ETB in hypoxia-induced PASMCs.

DISCUSSION

PH, a disease of the small pulmonary arteries, is a severe condition featured by abnormal smooth muscle cell growth and remodeling of the pulmonary vasculature (Humbert *et al.*, 2004). Although the pathogenesis is poorly understood, hypoxia is regarded as one of the key factors causing changes in pulmonary vessels. Under chronic hypoxic pulmonary circulation, smooth muscle cell proliferate and undergo hypertrophy making an important contribution to the vascular remodeling (Song *et al.*, 2013).

ET-1 is a powerful endothelium-derived vasoconstrictor and exerts a mitogenic effect on smooth muscle cells. Apart from proliferation, ET-1 has also been reported to cause inhibition of apoptosis in pulmonary arterial smooth muscles of rat (Jankov *et al.*, 2006). Findings from our previous studies have shown that a strong positive correlation between the elevated expression of ET-1 and the PH incidence in the broiler chickens exposed to high altitude hypoxia (Gao *et al.*, 2013). Several early reports have also suggested the up-regulation of ET-1 in the lungs of PH sufferer (Giaid *et al.*, 1993; Yamakami *et al.*, 1997), which can be down-regulated by using some ET antagonists attenuating the progress of PH in humans and animal models (Eddahibi *et al.*, 1995; Okada *et al.*, 1995). In present study, our observations further confirmed the idea that hypoxia for 24h significantly increased expression of ET-1 and its receptors mRNA as compared to the control group in avian PASMCs. We hypothesized that hypoxic exposure increases the ET production of PASMCs in broiler chickens and, as a result, causing PH and pulmonary vascular remodeling. Our present results are in agreement with those of Langleben *et al.* (1991) and Aguirre *et al.* (2000) who also suggested the increased ET-1 expression in response to the hypoxia in PASMCs and increased lung and circulating ET-1 levels during hypoxia resulting into pulmonary vascular remodeling.

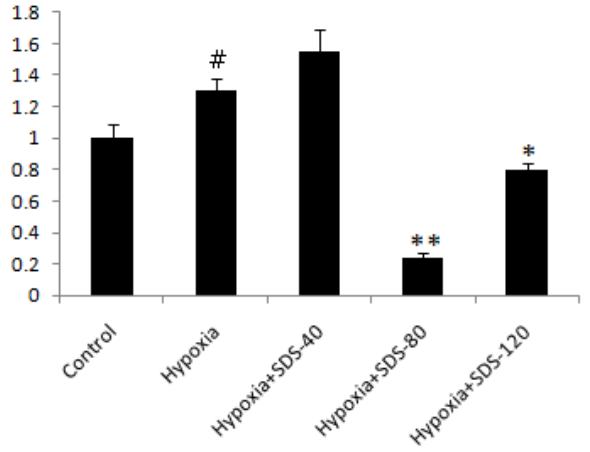

Fig. 3: qRT-PCR analysis demonstrating effects of different concentrations of SDS (40-1 20μg/ml) on ET-1 mRNA expression in avian PASMCs.

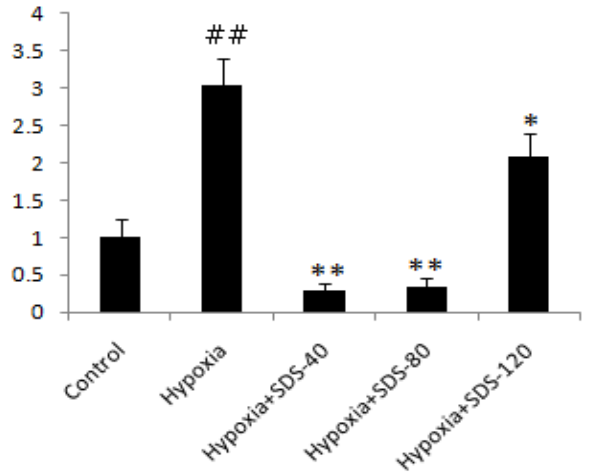

Fig. 4: qRT-PCR analysis demonstrating effects of different concentrations of SDS (40-120μg/ml) on ETA mRNA expression in avian PASMCs.

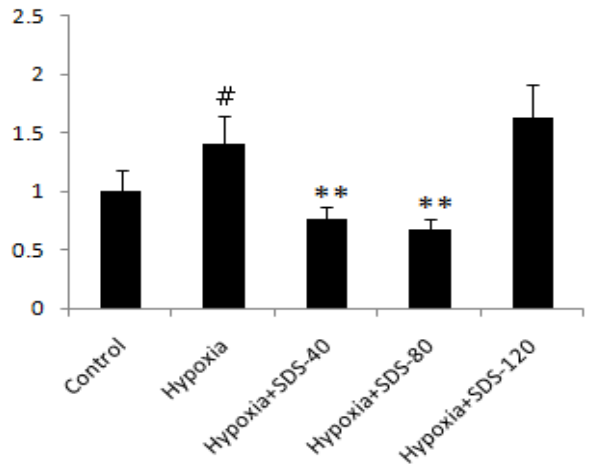

Fig. 5: qRT-PCR analysis demonstrating effects of different concentrations of SDS (40-120μg/ml) on ETB mRNA expression in avian PASMCs.

SDS, a major active ingredient occurring naturally in *Rhodiola rosea* L., has been widely employed for the treatment of fatigue, stress and mountain mal-hypoxia in Tibetan (Qian *et al.,* 2011). Here we designed the study to evaluate the pharmacological effect of SDS on expression of ET-1 and its receptors in hypoxia-treated PASMCs. Compared with the hypoxia-treated group, our data showed that among the various concentrations of SDS pretreatment, the levels of ET-1 gene expression were significantly decreased in both moderate and high dosage groups and no obvious decrease was observed in low doses, and these effects were found to be concentration dependent. At the same time, pretreatment with SDS at various concentrations was found to be effective in reducing the expression levels of ETA and ETB in PASMCs exposed to hypoxic conditions *in vitro*. These findings suggested that SDS can effectively attenuate hypoxia-induced lung injury by inhibiting ET-1 mRNA expression in response to hypoxia in avian PASMCs. One of the possible protective mechanisms of SDS might be due to its direct inhibition of ET produced by hypoxia, since SDS could attenuate hypoxia-induced up-regulation of BACE1 expression in SH-SY5Y cells (Li *et al.,* 2010). Similar findings have also been reported that SDS can promote angiogenesis by up regulating the expression of HIF-1α (Zhang *et al.,* 2009). Furthermore, in functional studies of Rhodiola extract, Liu *et al.* (2015) had elaborated that SDS can rescued mice from experimental sepsis via anti-inflammatory and anti-apoptosis effects.

Conclusions: The data obtained from the current study suggest that SDS can protect PASMCs against hypoxia-induced lung injury via regulating expression levels of ET in broiler chickens. The beneficial influence of SDS could be attributed to reduce expression levels of ET-1 and its receptors genes. These observations suggest another potential application value of SDS in the prevention and treatment of lung injury and ascites in broiler chicken production.

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Author's contribution: JL, LT and SY conceived and designed the experiments, analyzed the data and wrote the manuscript. JG and LT performed the experiments. LZ and QX contributed reagents/materials/analysis tools. All authors approved the manuscript and participated in revision work.

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