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

L Teng1,2, JF Gao1,2, L Zhou1, QY Xian1, JK Li1* and SJ Yang1*

1Center for Animal Experiment, Wuhan University, Wuhan 430071, China; 2College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China; 3Department of Pathology, Wuhan Medical Care Center for Women and Children, Wuhan 430016, China
*Corresponding author: 00030941@whu.edu.cn; lijk210@sina.com

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.

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 H2O2-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...
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 scraped 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/antimyotic for 2min. The tissue suspension was centrifuged at 500g for 4min and the supernatant was discarded. The pulmonary tissue was aspirated into 25cm² tissue culture flasks using a sterile glass Pasteur pipette and then placed in a CO₂ incubator for 3h. M199 complete culture medium (containing 15% FBS, 90U/ml heparin and 1% antibiotic/antimyotic) was added to infiltrate the pulmonary tissue and transferred into a 5% CO₂ 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 37°C with 5% CO₂. 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 37°C chamber containing 1% O₂ and 5% CO₂ 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.

Quantitative real-time-PCR (qRT-PCR) to evaluate the gene expression of ET-1, ETA and EBT 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'- CCTTCATTTGACC TTCACTACATGTCTA-3', and antisense, 5'- TGGAA GATGTTGATGCGCCCTTTCCATG-3'; ET-1, sense, 5'- GGAAGGGAGTGGCCTGTTAT-3', and antisense, 5'- GCTCCACGA AGCATCTCTG-3'; ETA, sense, 5'- GTGGCCCTTCTGGAGATTCTG-3', and antisense, 5'- GATTCCGATCTCCGTGAAACAC-3'; ETB, sense, 5'- CATCATCGACATCCCACATCA-3', and antisense, 5'- CACTATTTACATTTTCGACACCACA - 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: PASMC 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 with 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 with 120μg/ml. The expression of ET-1 was 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.
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. 4, 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 regulates the expression of ET 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.
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 contributed reagents/materials/analysis tools. All authors approved the manuscript and participated in revision work.

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