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# Chlorogenic acid inhibits hypoxia-induced pulmonary artery smooth muscle cells proliferation *via* c-Src and Shc/Grb2/ERK2 signaling pathway

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ABSTRACT

Chlorogenic acid (CGA), abundant in coffee and particular fruits, can modulate hypertension and vascular dysfunction. Hypoxia-induced pulmonary artery smooth muscle cells (PASMCs) proliferation has been tightly linked to vascular remodeling in pulmonary arterial hypertension (PAH). Thus, the present study was designed to investigate the effect of CGA on hypoxia-induced proliferation in cultured rat PASMCs. The data showed that CGA potently inhibited PASMCs proliferation and DNA synthesis induced by hypoxia. These inhibitory effects were associated with $G_1$ cell cycle arrest and down-regulation of cell cycle proteins. Treatment with CGA reduced hypoxia-induced hypoxia inducible factor 1α (HIF-1α) expression and *trans*-activation. Furthermore, hypoxia-evoked c-Src phosphorylation was inhibited by CGA. *In vitro* ELISA-based tyrosine kinase assay indicated that CGA was a direct inhibitor of c-Src. Moreover, CGA attenuated physical co-association of c-Src/Shc/Grb2 and ERK2 phosphorylation in PASMCs. These results suggest that CGA inhibits hypoxia-induced proliferation in PASMCs *via* regulating c-Src-mediated signaling pathway. *In vivo* investigation showed that chronic CGA treatment inhibits monocrotaline-induced PAH in rats. These findings presented here highlight the possible therapeutic use of CGA in hypoxia-related PAH.



## 1. Introduction

Lung vasculopathy is an irreversible pathologic hallmark of the lung vascular disorder pulmonary arterial hypertension (PAH), which is an often fatal and increasingly prevalent disease that is manifested by a maladaptive elevation of pulmonary vascular resistance and pulmonary arterial pressure (Grant et al., 2013). Current therapies for PAH provide a survival benefit, however, mortality rates still remain high and the treatment does not prevent the aggressive progression of the disease. As a result, newer treatments are required to more effectively manage PAH (Grant et al., 2013). Aberrant pulmonary artery smooth muscle cells (PASMCs) proliferation has been tightly linked to vascular remodeling in hypoxia-associated PAH. Thus, reduction of PASMCs proliferation using therapeutic agents may represent a promising strategy for the treatment of PAH.

c-Src is a cellular non-receptor tyrosine kinase which is originally identified as a proto-oncogene. Exposure to various stimuli (*e.g.*, hypoxia, platelet-derived growth factor (PDGF)) results in conformational change in protein structure of c-Src and its subsequent activation. Previous studies have demonstrated that hypoxic stimulation of VSMC is associated with significant upregulation in c-Src kinase activity and c-Src activation increased cellular proliferation leading to malignant transformation (Sato et al., 2005; Sayeski and Ali, 2003). Although the exact signal transduction mechanisms by which c-Src mediates these critical cellular events is not fully understood, evidence shows that the Shc/Grb2/ERK2 pathway plays a critical role in c-Src-mediated VSMCs proliferation (Sayeski and Ali,

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2003). Moreover, expression of HIF-1α, which is known to be responsible for the upregulation of hypoxia-sensitive gene products, also requires c-Src activation in hypoxia-stimulated VSMCs (Sato et al., 2005).

Chlorogenic acid (CGA), an ester of caffeic acid and quinic acid, is abundant in coffee and particular fruits (*e.g.*, berries, apples). CGA is considered to be one of the main bioactive phenolic acids in the diet and the daily intake is up to 1 g/day, especially for coffee-drinking population (Farah et al., 2008; Olthof et al., 2001). In recent years, CGA has attracted considerable interests due to its antibacterial, antioxidant, anticarcinogenic, hypoglycemic, and hypolipidemic activities, particularly anti-hypertension effects (Meng et al., 2013; Zhao et al., 2012). Basic and clinical investigations imply that the consumption of chlorogenic acid can modulate hypertension and vascular dysfunction (Mubarak et al., 2012; Suzuki et al., 2006a, 2006b; Zhao et al., 2012). These findings suggest the therapeutic potential of CGA as a novel strategy to treat cardio-related diseases. However, the role for CGA in modulating hypoxia-evoked aberrant PASMCs proliferation, which is of foremost physiological importance in hypoxia-associated PAH, is yet not elucidated.

Thereafter, the present study was designed to investigate the effects of CGA on hypoxia-induced PASMCs proliferation and the possible mechanisms involved. The results clearly show that CGA potently inhibited hypoxia-induced PASMCs proliferation, arrested PASMCs in G1 phase, and attenuated HIF-1α expression in PASMCs *via* direct depression of c-Src tyrosine kinase activity. Moreover, CGA modulated c-Src-mediated Shc/Grb2/ERK2 signaling pathway in PASMCs. *In vivo* study demonstrated that CGA treatment inhibits monocrotaline (MCT)-induced PAH in rats. Thus, our data presented here indicate the therapeutic potential of CGA in treating hypoxia-associated PAH.

## 2. Materials and methods

### 2.1. Materials and reagents

Chlorogenic acid is a reference compound (purity ≥ 98.0%) supplied by the Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Ministry of Public Health, China. PP1 was from Biomol (Plymouth Meeting, PA, USA). Monocrotaline, DCFH-DA and BrdU were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal anti-β-actin, monoclonal anti-phospho-ERK1/2, polyclonal anti-HIF-1α, polyclonal antibody to ERK1/2 and immunoprecipitating anti-Src polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to Shc and immunoprecipitating anti-Src monoclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Antibody to α smooth muscle actin (α-SMA) was purchased from Boster Biotechnology (Wuhan, Hubei, China).

### 2.2. Cell culture and treatment

PASMCs were obtained from the pulmonary arteries of male Sprague–Dawley rats weighing 180–220 g using the tissue explant method, as described previously (Lin et al., 2007). The experimental protocol was approved by the local ethics committee. Cells were grown in DMEM supplemented with 10% FBS in 5% $CO_2$ and 95% air at 37 °C (Normoxic condition, 21% $O_2$). More than 98% of the cells were positive for smooth muscle-specific α-actin, and exhibited the typical morphology of PASMCs. Cells between passages 2 and 5 were used in these studies to ensure the genetic stability of the culture.

### 2.3. Hypoxia (3% $O_2$)

PASMCs were placed in a modular chamber (Billups Rothenberg, Del Mar, CA, USA) and flushed with a mix of 0% $O_2$, 5% $CO_2$, and 95% $N_2$ at 12 l/min for 20 min. Chambers remained tightly sealed and then were placed in a 5% $CO_2$, 37 °C incubator (Chanakira et al., 2012). A blood gas analyzer was used to detect the oxygen tension ($PO_2$) from cell culture medium to ensure that the hypoxic conditions were achieved (Chanakira et al., 2012).

### 2.4. Cell proliferation assay

The cell proliferation assay was performed using the MTT method as described previously (Chen et al., 2009). Briefly, PASMCs were treated under normoxia or hypoxia for 48 h, and then the cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C. Finally the culture medium was removed, and the Formazan salt crystals were dissolved with 200 μl dimethylsulfoxide (DMSO) and shaken for 10 min. The absorbance was read at a wavelength of 570 nm using Spectramax M2 microplate Reader (Molecular Devices). To validate the MTT assay, trypan blue exclusion was performed in parallel.

### 2.5. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

DNA synthesis in PASMCs was examined using the BrdU incorporation assay according to the method described previously (Sasu et al., 2001). 10 μmol/l BrdU was added to PASMCs which have been treated under normoxia or hypoxia for 48 h. To immunostain BrdU, the cells were washed with PBS, fixed in 4% polyformaldehyde and then permeabilized with 0.1% Triton X-100. After DNA denaturation with 4 mol/l HCl, non-specific binding sites were blocked with 5% non-fat milk. The cells were then stained with antibody for BrdU followed by incubation with the Alexa Flour 568 goat-anti-mouse IgG (Invitrogen) secondary antibody. The cell nuclei were stained with Hoechst 33342 and evaluated by fluorescence microscopy with the appropriate fluorescent filters. Results are presented as mitotic index, and defined as the percentage of BrdU-positive nuclei per number of cells.

### 2.6. Flow cytometry analysis of cell cycle

Rat PASMCs were trypsinized, collected, and washed twice with cold PBS. Cells pellets were fixed in 70% ethanol and stored at 4 °C. Next, the fixed cells were treated with RNase A (10 μg/ml). DNA was stained with propidium iodide (50 μg/ml) for 30 min at 37 °C, and $1 \times 10^4$ cells were analyzed by flow cytometry. The rates of $G_0/G_1$, S and $G_2/M$ phases were determined using the computer program ModiFit LT.

### 2.7. Assessment of intracellular reactive oxygen species production

Reactive oxygen species production was quantified by the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) method (Chen et al., 2009), based on the reactive oxygen species-dependent oxidation of DCFH-DA to 2′,7′-dichlorofluorescein (DCF). PASMCs grown in 96-well plates were then incubated with DCFH-DA (10 μmol/l) for 30 min. Fluorescence of the samples was monitored at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

### 2.8. Glutathione-S-reductase (GST) enzyme activity assay

The activity of GST, the typical phase 2 enzyme, was measured spectrophotometrically as described previously (Feng et al., 2005). PASMCs were lysed with 200 μl of lysis buffer (0.25 M sucrose,