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Carvacrol induces the apoptosis of pulmonary artery smooth muscle cells under hypoxia

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ABSTRACT

The abnormal apoptosis of pulmonary artery smooth muscle cells (PASMCs) is an important pathophysiological process in pulmonary vascular remodeling and pulmonary arterial hypertension (PAH). Carvacrol, an essential oil compound from oregano and thyme, has displayed antimicrobial, antitumor, and antioxidant properties. Although carvacrol has pro-apoptosis properties in tumor cells, the underlying mechanisms of carvacrol in PASMC apoptosis remain unclear. Thus, in this study, we aim to investigate the role of carvacrol in pulmonary vascular remodeling and PASMC apoptosis in hypoxia. Right Ventricular Hypertrophy Measurements and pulmonary pathomorphology data show that the ratio of the heart weight/tibia length (HW/TL), the right ventricle/left ventricle plus septum (RV/LV+S) and the medial width of the pulmonary artery increased in chronic hypoxia and were reversed by carvacrol treatment under hypoxia. Additionally, carvacrol inhibited PASMC viability, attenuated oxidative stress, induced mitochondria membrane depolarization, increased the percentage of apoptotic cells, suppressed Bcl-2 expression, decreased procaspase-3 expression, promoted caspase-3 activation, and inhibited the ERK1/2 and PI3K/Akt pathway. Taken together, these findings suggest that carvacrol attenuates the pulmonary vascular remodeling and promotes PASMC apoptosis by acting on, at least in part, the intrinsic apoptotic pathway. This process might provide us new insight into the development of hypoxic pulmonary hypertension.

1. Introduction

Pulmonary arterial hypertension (PAH) is a complex disease that is clinically characterized by an increase in pulmonary vascular resistance leading to right heart failure and death (Farber and Loscalzo, 2004; McGoon and Kane, 2009; Stenmark et al., 2006). The histopathology of PAH is marked by sustained vasoconstriction, the thickening of pulmonary artery walls, vascular remodeling and vessel obstruction (Chan and Loscalzo, 2008). The pulmonary vascular remodeling in PAH is characterized by pulmonary vascular medial hypertrophy and lumen narrowing, which is mainly caused by imbalanced proliferation and apoptosis in pulmonary artery smooth muscle cells (PASMCs). Because the inhibition of PASMCs apoptosis is an essential feature of pulmonary hypertension, considerable efforts have been made to develop a therapeutic strategy that effectively induces PASMC apoptosis. Recently, 'apoptosis-based therapeutic strategies' to reduce pulmonary vascular thickening and vascular remodeling have gained attention and have been used successfully in experimental animal models (Brevnova et al., 2004; McMurtry et al., 2004; McMurtry et al., 2005; Merklinger et al., 2005; Nishimura et al., 2003). Thus, the augmentation of PASMC apoptosis could serve as therapeutic approach for patients with PAH.

Extracellular signal-regulated kinase 1/2 (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family, is one of the most frequently studied signaling systems. ERK1/2 inhibits apoptosis and promotes the survival of vascular smooth muscle cells (Wang et al., 2002). The activation of the ERK1/2 and PI3K/Akt signal pathway is involved in the anti-apoptosis process of PASMCs (Li et al., 2006; Wang et al., 2002). Akt is a serine/threonine protein kinase that is activated by a number of growth factors and cytokines in a PI3K-dependent manner. PI3K/Akt also inhibits cellular apoptosis and promotes cell survival in response to growth factor

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induction (Gerasimovskaya et al., 2005; Shiojima and Walsh, 2002).

Carvacrol (5-isopropyl-2-methylphenol), a monoterpenoid phenol, is one of the main substances of the essential oil from oregano and thyme (Kiskó and Roller, 2005; Lampronti et al., 2006). Carvacrol has well-known antibacterial, antiviral, insecticidal and antioxidant properties (Sökmen et al., 2004) and has generally been recognized as a safe food additive and a cosmetic ingredient (Burt, 2004). Previous studies revealed that this compound suppresses the growth of mouse B16 melanomas cells (He et al., 1997) and human metastatic breast cancer cells and inhibits DNA synthesis in mouse myoblasts bearing a human N-ras oncogene (Zeytinoglu et al., 2003), suggesting that carvacrol may act as an anticancer agent. Carvacrol has pro-apoptosis properties in the non-small-cell lung cancer cell line A549, the intestinal cells line CaCo-2 and human glioblastoma cells (Fabian et al., 2006; Koparal and Zeytinoglu, 2003; Liang and Lu, 2012; Llana-Ruiz-Cabello et al., 2014). However, the underlying molecular mechanisms of carvacrol in PASMC apoptosis remain unclear. Therefore, in this study, for the first time, we tried to investigate the role of carvacrol in PASMC apoptosis under hypoxia and determine whether the ERK1/2 and PI3K/Akt pathway participates in the process of carvacrol-induced PASMC apoptosis.

2. Materials and methods

2.1. Materials

Carvacrol and all of the other reagents were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against procaspase-3, Bcl-2, bax, caspase-3, ERK1/2 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Alpha-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). JC-1 probe, Rhodamine 123, Hoechst 33342, the caspase-3 activity kit and the Annexin V-FITC Apoptosis Detection Kit were purchased from the Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham International (Amersham, UK). All of the other reagents were from common commercial sources.

2.2. Animal and lung tissue preparation

Adult male Wistar rats (150–200 g) were used in this study. The rats were from the Animal Research Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC), at a controlled ambient temperature of 22 ± 2 °C with (50 ± 10)% relative humidity and a 12-h light-dark cycle (lights on at 8:00 AM). Adult Wistar rats were randomized to 9 days of normal and hypoxic environments with fractional inspired oxygen (FiO_2) at 0.21 and 0.12, respectively, as previously described (Zhu et al., 2003). Normoxic rats were kept in the same room adjacent to the hypoxic chamber. The animals were randomly divided into five groups: (1) normoxia group ($n=6$), which received the vehicle intraperitoneally (physiological saline 0.1 ml/100 g); (2) chronic hypoxia group ($n=6$), which received the vehicle intraperitoneally (physiological saline 0.1 ml/100 g); and (3–5) carvacrol groups ($n=6$), which at the beginning of hypoxia exposure were treated with 25, 50 and 100 mg/kg carvacrol via intraperitoneal injection once daily lasting for 9 days. At the end of the 9-days exposure period, we anesthetized each rat with pentobarbital injection (120 mg/kg, i.p.), opened the thorax and removed the heart and lungs to a flat plate. In some experiments, the lungs were quickly removed and further processed for hematoxylin and eosin (HE) staining as described below.

2.3. Right ventricular hypertrophy measurements

After 9 days of hypoxia or normoxia, the rats were weighed and anesthetized and we opened the thorax and removed the heart and lungs to a culture plate. The heart was weighed, carefully dissected into right ventricle (RV), left ventricle (LV) and septum (S), and individually weighed. The degree of right ventricular hypertrophy was assessed by measuring the ratio of the heart weight/tibia length (HW/TL) and the ratio of right ventricle/left ventricle and septum (RV/LV+S).

2.4. Histology

The lung tissues were obtained from anesthetized rats, sliced into tissue blocks, and immersed in 4% paraformaldehyde for overnight fixation. The fixed tissues were then dehydrated, cleared, and embedded in paraffin wax. The tissues were cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E). The results were expressed as the ratio of wall thickness/vessel radius. The sections were viewed with an Eclipse 600 Nikon microscope and photographed with a digital camera. Morphometric analysis was performed with image software (Image Pro Plus).

2.5. Cell culture

The PASMCs were prepared according to our previously published protocol (Guo et al., 2008). The cell viability as determined by Trypan Blue exclusion was consistently greater than 98%. The purity of PASMCs in the primary cultures was confirmed by the specific monoclonal antibody that was raised against smooth muscle α-actin. The cells were cultured in 20% fetal bovine serum (FBS)-DMEM in a 37 °C, 5% CO_2 humidified incubator. Cells under hypoxic condition were incubated with a gas mixture containing 3% O_2, 5% CO_2, and 92% N_2 for 24 h. Passages 2–4 were used for further experimentation.

2.6. MTT assay

PASMCs were cultured in 96-well microtitration plates (approximately 1×10^4 per well) and then were subjected to growth arrest for 24 h. Carvacrol wes diluted first in ethanol and then filtered through a Millipore filter (Millex-GS 0.22-μm pore size; Millipore, Ireland) and subsequently diluted in DMEM. The same amount of filtered ethanol was added to DMEM in every control group (10 μl/ml). The cells were treated with carvacrol at different concentrations under normoxic and hypoxic conditions. After 24 h of incubation at 37 °C, the cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). After removing the supernatant, dimethyl sulfoxide (150 μl/well) was added. The plates were then agitated on a plate shaker for 10 min at room temperature. The absorbance was measured at 490 nm in a spectrophotometer.

2.7. Measurement of malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione (GSH) activities

The enzymatic activities of SOD, GSH and MDA were measured according to the manufacturer's instructions using different commercially assay kits (Beyotime Institute of Biotechnology, China).

The concentration of MDA was determined in PASMCs by measuring thiobarbituric acid (TBA)-reacting substances at a wavelength of 532 nm. The level of MDA was expressed as nanomoles of MDA per milligram of protein. The SOD activity was estimated by calculating the rate inhibition of nucleotide oxidation. The results were defined as units per milligram of protein. The activities