Carnosol Is a Potent Lung Protective Agent: Experimental Study on Mice

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ABSTRACT

Introduction. Oxidative stress has been implicated in various disease states and ischemia/reperfusion injury is a direct consequence of oxidative stress in lung transplantation. Because the success rate of organ transplantation in which ischemia/reperfusion is inevitable is highly influenced by oxidative stress, development of strategies to control oxidative stress would be beneficial. Here we identified natural compounds to reduce oxidative stresses in isolated mouse lungs.

Methods. We screened compounds associated with antioxidative stress in 200 plant extracts by monitoring the activities of nuclear factor erythroid 2-related factor 2 (NRF2). Compounds found to ameliorate antioxidative stress were enriched and mice were administered the extract orally every day for 1 week. Then, the lungs were isolated and cultured in the culture medium at 37°C. Lung damage was monitored by lactate dehydrogenase (LDH) released in the culture medium. Arterial (left ventricle) blood gas levels were also monitored after hilar clamping.

Results. We found that *Callicarpa longissima* extract was rich in NRF2 activators. The responsible compounds were carnosic acid and its oxidative product, carnosol. Carnosol induced heme-oxygenase 1 (HO-1) expression, which is downstream of NRF2, more efficiently than carnosic acid.

Conclusions. Lungs from mice treated with *C longissima* extract were less damaged than those from control mice and accompanied by HO-1 induction. These results suggest that carnosol is a candidate compound to increase the success rate of lung transplantation.

LUNG transplantation is an established treatment for end-stage lung disease under refractory and inescapable conditions [1]. However, despite the increasing number of lung transplantations annually, the overall outcomes remain unsatisfactory as compared with reports of other solid-organ transplantation settings [2].

Oxidative stress induced by reactive oxygen and nitrogen species is thought to be involved in various disease states, including organ transplantation, which is associated with ischemia/reperfusion injury and activation of inflammation and other immune responses, resulting in potential organ rejection and bronchiolitis obliterans syndrome, among other postoperative complications [3,4]. Therefore, development of a novel, efficient, and comprehensive therapeutic strategy to successfully abrogate the effects of oxidative stress could significantly improve short-term and long-term outcomes of lung transplant recipients.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor that mediates a broad-based set of

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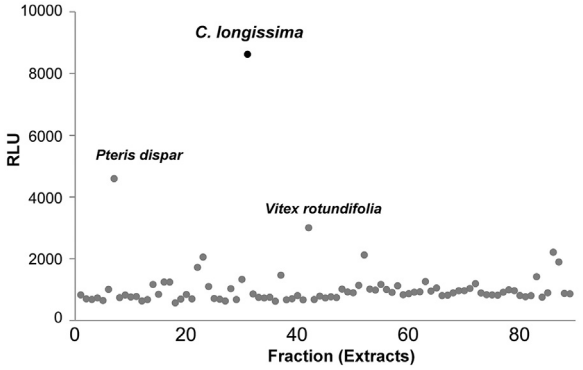

Fig 1. Screening of NRF2 activator. Primary fibroblasts coding for the OKD48-luc reporter were plated on 96-well plates. After 24 h, plant extracts (100 μg/mL) were added to the culture medium and the cells were incubated for an additional 3 h. This evaluation was performed as an independent duplicate and representative data from plate 1 are shown. Abbreviation: RLU, relative luciferase unit.

adaptive responses to endogenous and exogenous oxidative stressors by inducing expression of a number of antioxidant and cytoprotective factors, including heme oxigenase-1 (HO-1), through promoter activation via the antioxidant response element [5]. Under nonstressed conditions, kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1) recruits NRF2, and the resultant complex is rapidly diverted to a degradation pathway via ubiquitination, the so-called proteasomal pathway. Once cells are subjected to oxidative stresses, thiol moieties in the KEAP1 protein are oxidized, thereby triggering the dissociation of KEAP1 from NRF2, resulting in the stabilization and accumulation of NRF2. Thus, modulators of KEAP1–NRF2 complex formation may also induce antioxidant activities.

Carnosic acid and its oxidative product carnosol are plant-derived compounds that function as antioxidants [6]. In addition to their chemical properties, these molecules have been shown to stabilize NRF2 via the modulation of KEAP1–NRF2 complex formation. In the present study, we identified a natural source rich in carnosic acid and carnosol, *Callicarpa longissima*, to determine whether carnosol-enriched extract conveyed a protective effect on lung tissues during warm ischemia.

MATERIALS AND METHODS

Plant Extracts and Chemicals

Plant materials were obtained from fields of the Tanegashima and Tsukuba Divisions of the Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, and then dried for 2–3 days at 50°C (LC-234 Dryer; ESPEC Corp., Tokyo, Japan) and ground to flour. Then, 5 g of the resultant powder was soaked in 20 mL of ethanol for 2 days and the extract was passed through a 2-mL activated charcoal filter (Wako Pure Chemicals Co., Ltd., Kyoto, Japan) to exclude chlorophyll and related compounds. Ethanol was evaporated and the extracts were resuspended in ethanol at 10 mg/mL. Assay-grade carnosol and carnosic acid were purchased from Wako Pure Chemicals Co., Ltd. Anti-NRF2 and -HO-1 antibodies were purchased from Abcam Plc. (Cambridge, Mass, United States), and antiglyceraldehyde 3-phosphate dehydrogenase antibody was obtained from Cell Signaling Technology Japan, KK (Tokyo, Japan). Antigen-antibody interactions were detected using ECL chemiluminescence reagent (GE Healthcare, Uppsala, Sweden) and then visualized using the Bio-Rad ChemiDoc Imager (Bio-Rad Laboratories KK, Tokyo, Japan), or detected by a chromogenic reaction using the Immunostain HRP kit (Atto Co., Ltd., Tokyo, Japan). Lactate dehydrogenase (LDH) levels were measured using a WST-8 kit (Wako Pure Chemicals Co., Ltd., Osaka, Japan) and a DRY-CHEM 3500S chemical analyzer (Fujifilm, Tokyo, Japan).

Mice and Cell Culture

OKD48-luc transgenic mice were purchased from TransGenic Inc. (Kobe, Japan). C57BL/6J mice were purchased from SLC Japan (Shizuoka, Japan). The experimental mouse protocols were approved by the ethics committee of the National Institute of Biomedical Innovation (approval no. DS24-44). The animals were maintained under standard conditions of light (08:00–20:00 hours) and temperature (23°C; 60% humidity).

Primary fibroblasts were prepared from the skin of neonates of OKD48-luc transgenic mice. For screening, 10^5 cells/well after the third passage were cultured in 96-well plates with 100 μL of Dulbecco's Modified Eagle Medium (DMEM). After 24 hours, an additional 50 μL of DMEM containing plant extracts were added to each well and the plates were incubated for an additional 3 hours before luciferase activity was measured using a luciferase reagent (Promega KK, Tokyo, Japan). Human lung non–small cell carcinoma NCI-H1975 cells were obtained from the American Type Culture Collection (Manassas, Va, United States) and maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Preparation of *C longissima* Extract

The procedure for *C longissima* extract preparation is described elsewhere. Briefly, 40 g of dried powder of leaves of *C longissima* were soaked in 400 mL of ethanol. After 3 extractions, the final extract was concentrated to 40 g/100 mL by evaporation and then passed through 40 g of activated charcoal. To exclude water-soluble compounds, 100 mL of hexane was added to the flow-through fraction, and the viscous solution (approximately 5 mL) was discarded. Ethanol and hexane were evaporated, and the extract was dissolved with chloroform and applied into a silica gel column (100 mL; Nacalai Tesque, Inc., Kyoto, Japan). Substances suppressing melanin production were eluted from the solvent using chloroform and methanol at a ratio of 9:1. The resultant fraction contained carnosol, but not carnosic acid, with a purity of >90%, as confirmed

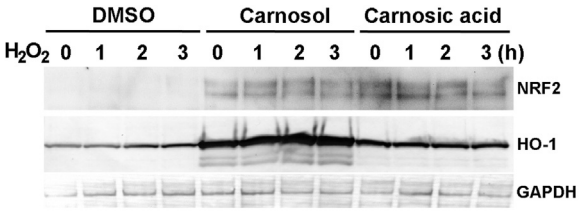

Fig 2. Carnosol induces HO-1 protein. NCI-H1975 cells (1×10^5) were plated on a 12-well plate and cultured until confluence. Then, the medium was changed with freshly prepared medium supplemented with carnosol or carnosic acid (3 μmol/L). After 1 h, the cells were subjected to H_2O_2 (10 μmol/L) for 0–3 h and lysed for Western blotting. Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.