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Research Article
Nootkatone from the Rhizomes of *Cyperus rotundus* Protects Against Ischemia-reperfusion Mediated Acute Myocardial Injury in the Rat

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Abstract

Background and Objective: The up-regulation of heme oxygenase-1 (HO-1) has been reported to protect from ischemia-reperfusion (I/R) injury and nootkatone, a pharmacologically active ingredient found in the rhizomes of *Cyperus rotundus* has been reported to induce HO-1 in immune cells. The aim of the present study was to determine whether the protective effect of *C. rotundus* extract and its constituent, nootkatone against myocardial I/R injury is due to its antioxidant and anti-inflammatory effects. **Materials and Methods:** Nootkatone was isolated from the rhizomes of *Cyperus rotundus* using chromatographic techniques. The concentration of nootkatone in the extract was analyzed by HPLC to be 0.254±0.155 μg mg^{-1}. Adult male rats were subjected to 30 min of ischemia and 24 h of reperfusion. Rats were randomized to receive vehicle, extract (10 mg kg^{-1}) or nootkatone (5 or 10 mg kg^{-1}) 1 h before reperfusion. Infarct sizes were measured and myocardial functions assessed. **Results:** Nootkatone and the extract of *C. rotundus* at 10 mg kg^{-1} significantly ameliorated I/R-induced myocardial dysfunction by increasing the first derivative (±dp/dt) of left ventricular pressure and decreasing infarct size. **Conclusion:** The present study suggests that the extract of *C. rotundus* rhizomes and its active ingredient, nootkatone protect heart from I/R injury by reducing oxidative stress and the expressions of inflammatory mediators by HO-1 induction.

Key words: Nootkatone, *Cyperus rotundus*, heme oxygenase-1, ischemia-reperfusion, myocardial injury, rat heart, isolation, quantitative analysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Myocardial infarction is a common type of ischemic heart disease, which is the leading cause of disease-related death worldwide. In the ischemic heart, cardiac damage is initiated by a diminished blood supply and swift restoration of blood supply is imperative to minimize cardiac injury. However, reperfusion itself can induce additional injury in the form of cardiac dysfunction, reperfusion arrhythmia and by exacerbating myocardial infarction. Oxidant stress seems to play a major role in the organ injury that occurs during ischemia and reperfusion (I/R)[1]. Infact, elevated Reactive Oxygen Species (ROS) levels and the accumulation of calcium in cytosol and mitochondria are causative factors of I/R injury[2]. Furthermore, heme oxygenase-1 (HO-1) up-regulation has been reported to protect the liver[3], heart[4] and kidneys[5] from I/R injury. These findings suggest that the induction of HO-1 might attenuate I/R-induced injury by inducing anti-inflammatory and anti-oxidant effects. Previously, it was reported that nootkatone, an active constituent of the rhizomes of *Cyperus rotundus*, induced HO-1 and increased survival rates in a mouse cecal ligation and puncture (CLP)-induced model of sepsis[6]. Furthermore, nootkatone has been shown to inhibit chemokine induction by inflammatory cytokines in HaCaT cells[7] and to prevent diet-induced obesity by activating 5'-adenosine monophosphate-activated protein kinase (AMPK)[8]. Therefore, the purpose of the present study was to investigate the hypothesis that nootkatone might protect rats from heart I/R-injury.

MATERIALS AND METHODS

Extraction and isolation: The rhizomes of *Cyperus rotundus* were purchased from an oriental market place (Kyung Dong, Seoul, Korea) and identified by Prof. Je-Hyun Lee, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. A voucher specimen (DKH-02561) was deposited at the Ministry of Food and Drug Safety, Korea. The preparation of 70% EtOH extract of the rhizomes of *C. rotundus* and the isolation of nootkatone (Fig. 1) were performed according to our previous method[9].

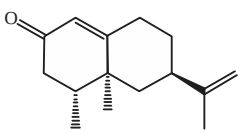

Fig. 1: Chemical structure of nootkatone

Identification and quantitative analysis: The structure of nootkatone was identified by NMR and LC-MS measurements. The peak purity and quantitative analyses of nootkatone in the total extract was performed by LC/HR-ESI-MS techniques using LC/Q-TOF mass spectrometer (UHPLC/1290 Infinity; Agilent, Waldbronn, Germany). The HPLC separation was performed using RP C18 column (Zorbax Eclipse Plus, 2.1×50 mm, 1.8 μm). The mobile phase was prepared from water-formic acid 0.1% (solvent A) and acetonitrile-formic acid 0.1% (solvent B) in a gradient elution, starting with 5% B, increasing to 90% B (0-14 min), held at 90% B and decreasing to 5% B (17-25 min) with flow rate of 0.3 mL min^{-1}. The MS analysis was achieved using Dual AJS ESI ion source with the scan rate of 1.5 spectra per second and positive polarity (4 kV).

Experimental animals: Male Sprague-Dawley rats (7-8 weeks old) were used in this study. All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH publication No. 85-23; 1996 revision). The study protocol was approved beforehand by the Care of Animals in Research Committee of Gyeongsang National University, Republic of Korea. The rats were randomly distributed into four groups: Group 1 (Sham): Sham-operated (24 h after surgery, n = 8) rats in which no tightening of the coronary artery sutures was performed, group 2 (I/R): Rats pretreated with a placebo (saline, 0.25 mL, i.p., injection 1 h before ischemia) and subjected to 30 min of ischemia followed by a 24 h (n = 10) period of reperfusion, group 3 (I/R+nootkatone): Rats pretreated with nootkatone (i.p., injection 1 h before ischemia) and subjected to 30 min of ischemia followed by 24 h (5 mg kg^{-1}, n = 8; 10 mg kg^{-1}, n = 10) period of reperfusion and group 4 (I/R+extract): Rats pretreated with the extract (10 mg kg^{-1}, i.p., injection 1 h before ischemia) and subjected to 30 min of ischemia followed by a 24 h (n = 10) period of reperfusion.

Surgical preparation: Surgery was performed under zoletil (25 mg kg^{-1}) and xylazine (10 mg kg^{-1}) general anesthesia, endotracheal intubation using a 14 G angiocatheter and mechanical ventilation and pericardiotomy by opening the chest along the left side of the sternum through the ribs. A 4-0 black silk on a curved tapered needle was then passed below the left descending vein and coronary artery from immediately below the left atrial appendage to the right portion of the left ventricle and its ends were passed through a small vinyl tube to form a snare. Tightening the snare occluded the coronary artery branch and this was fixed by clamping the tube with a

small hemostat. Occlusion was verified by epicardial cyanosis. After occlusion for 30 min, the snare was released and the tube was removed to initiate reperfusion (24 h), which was confirmed by epicardial hyperemia. The loosened suture was left in place to allow the ischemic area to be evaluated. The extract, nootkatone or vehicle (saline) was treated 1 h prior to I/R injury intraperitoneally (i.p.) as a bolus dose.

Hemodynamic measurements: After 24 h of reperfusion, rats were anesthetized with zoletil (12.5 mg kg^{-1}) and xylazine (7.5 mg kg^{-1}). The right common carotid artery was exposed and cannulated with a 2 F Millar Catheter (Millar Instruments, USA) into the ascending aorta to measure systolic and diastolic blood pressure, MAP and HR. The pressure transducer was then advanced into the LV to measure LV systolic, LVEDP and the first derivatives (positive and negative) of LV pressure (±dp/dt).

Determination of AAR and infarct size: After obtaining hemodynamic measurements, the LAD coronary artery was re-occluded at the same site and 2 mL of 1% evans blue dye was injected into the inferior vena cava to allow perfused and non-perfused (AAR) heart regions to be differentiated. The AAR was then cut into small pieces and incubated in a 1% solution of 2, 3, 5-triphenyltetrazolium chloride stain for 20 min at 37°C, to visualize the IA. Stained and unstained regions were separated and weighed to determine infarct size, which was calculated by expressing the weight of the unstained region as a percentage of AAR weight.

Statistics: Data are expressed as the Mean±SD of results obtained from the number of replicate treatments. Differences between data sets were assessed by one-way analysis of variance followed by Newman-Keuls tests. The $p<0.05$ was accepted as statistically significant.

RESULTS

Hemodynamics: Left ventricular end-diastolic pressure (LVEDP), positive and negative maximum values of the first derivatives of left ventricular pressure (±dP/dt), Heart Rate (HR) and Mean Arterial Pressure (MAP) were investigated at 24 h after reperfusion. Table 1 shows a marked elevation in LVEDP was observed in the I/R group (12±2 mmHg) and this was significantly ($p<0.05$) reduced by the extract of *C. rotundus* rhizomes (6±2 mmHg) and nootkatone (7±2 mmHg) at a dose of 10 mg kg^{-1}. However, nootkatone at 5 mg kg^{-1} showed a weak effect with no significance. The ±dP/dt were significantly improved by the extract and nootkatone versus the I/R control. Other parameters, such as, MAP and HR were not significantly different in the extract, nootkatone and I/R groups. However, MAP was significantly decreased by I/R ($p<0.05$).

Area at risk and infarct size: Since nootkatone improved myocardial function, infarct sizes were measured. The area at risk (AAR)/left ventricle (LV), infarct area (IA)/LV and IA/AAR values are displayed in Fig. 2. The ischemic areas induced by the Left Anterior Descending (LAD) snare (AAR/LV, %) were similar in the groups. However, IA/LV and IA/AAR were significantly lower in the nootkatone group at 10 mg kg^{-1} than in the I/R group. However, nootkatone at 5 mg kg^{-1} revealed no significant effect compared to I/R

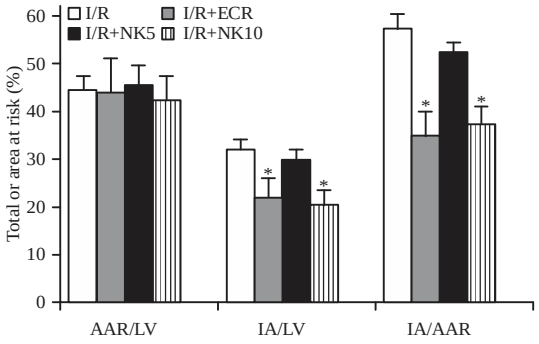

Fig. 2: Measurements of myocardial infarct sizes in rat hearts subjected to I/R injury. ECR: Total extract of *C. rotundus* rhizomes (10 mg kg^{-1}), NK5 or NK10 (nootkatone 5 or 10 mg kg^{-1}, respectively), I/R: Ischemia and reperfusion, LV: Left ventricle, IA: Infarct area, AAR: Area at risk. Results are Means±SD. *$p<0.05$ vs. I/R

Table 1: Summary of hemodynamic measurements in myocardial ischemia/reperfusion

Parameters	LVSP/LVEDP (mmHg)	±dP/dt (mmHg sec^{-1})	HR (bpm)	MAP (mmHg)
Sham (n = 3)	105±9/4±1	3847±228/-3058±135	274±25	82±4
I/R (n = 4)	87±3†/12±2†	3018±280†/-2369±164†	294±24	68±4†
Extract (n = 3)	98±5*/6±2*	3833±247*/-2911±255*	267±13	73±6
NK5 (n = 3)	96±6*/11±1	3388±329/-2533±276	261±15	72±5
NK10 (n = 4)	98±6*/7±2*	3813±257*/-2912±249*	265±15	73±4

LVSP: LV systolic pressure, LVEDP: LV end-diastolic pressure, dP/dt: First derivatives of LV pressure over time, MAP: Mean artery pressure, HR: Heart rate, results are expressed as Means±SD, †$p<0.05$ vs. Sham, *$p<0.05$ vs. I/R. NK5 and NK10: Nootkatone 5 and 10 mg kg^{-1}, respectively. Dose of the extract: 10 mg kg^{-1}

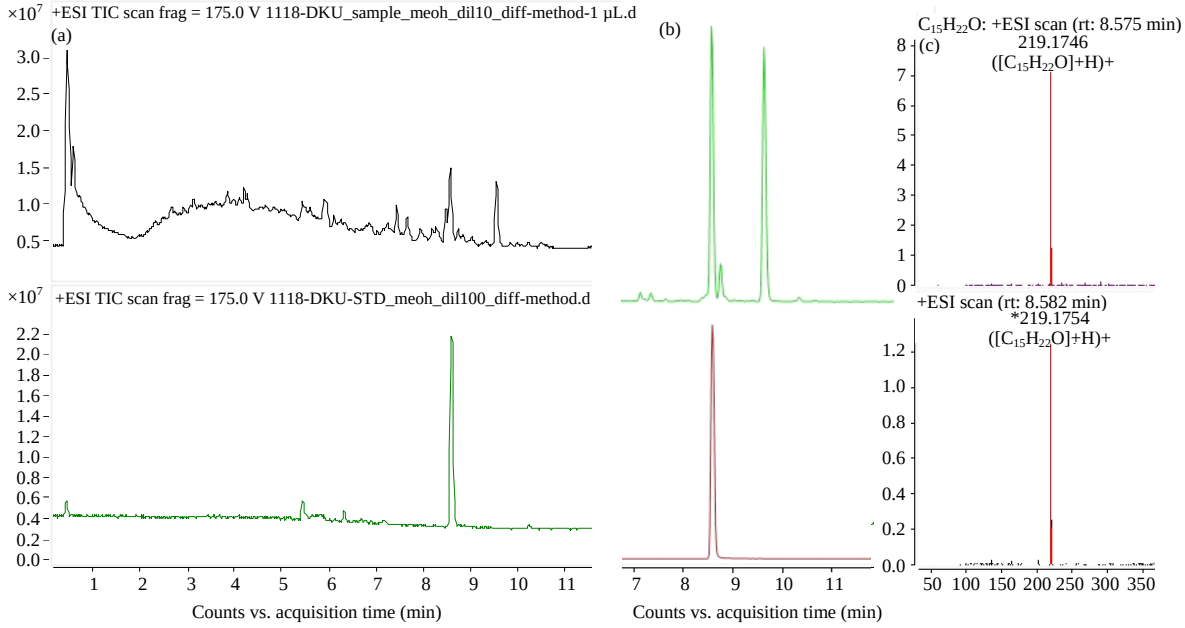

Fig. 3(a-c): Qualitative and quantitative analyses of nootkatone in the extract of rhizomes of *C. rotundus*. (a) HPLC total ion chromatogram of the extract (upper) and nootkatone (Rt = 8.582 min, below), (b) HR-ESI-MS spectrum of m/z 219.1754 peak (MH^+) for $C_{15}H_{22}O$ extracted from the total ion chromatogram providing three compounds (upper) and nootkatone (below) and (c) The identity of both peaks at Rt = 8.582 min was further confirmed by their HR-MS data

group. As expected, the extract of *C. rotundus* also significantly reduced those parameters (IA/LV, IA/AAR) compared to I/R group.

Qualitative and quantitative analyses of nootkatone: The HPLC chromatograms of the total extract (upper) and nootkatone (Rt = 8.582 min, below) were presented in Fig. 3a. The MH^+ peaks at m/z 219.1754 detected by HR-ESI-MS were extracted from the total ion chromatogram to give 3 compounds with the molecular formula of $C_{15}H_{22}O$ (Fig. 3b, upper), among them, the biggest peak was exactly consistent with that of nootkatone (Fig. 3b, below). The identity of both peaks at Rt = 8.582 min was further confirmed by their HR-MS data (Fig. 3c), proving that the peak of nootkatone in the extract is pure (98% purity). The HPLC method shows linearity over the range of 12.5-200 µg mL^{-1} and correlation coefficient of 0.9990. The concentration of nootkatone in the extract was 0.254±0.155 µg mg^{-1}.

DISCUSSION

The genus *Cyperus* includes plants commonly found in uplands and paddy fields in temperate to tropical regions. In Asia, the rhizomes of *C. rotundus* have been used as a traditional folk medicine to treat stomach and bowel disorders and inflammatory diseases. Recently, some studies demonstrated that *C. rotundus* protected brain hypoxia injury[10] and ischemia-induced brain damage in rats[11]. Importantly, some active ingredients of *C. rotundus*, such as oleanolic acid, showed cardioprotective action in hyperglycemia-induced contractile dysfunction[12], indicating that *C. rotundus* has beneficial effect in cardiovascular system. It is found that pretreatment with the extract of *C. rotundus* and nootkatone significantly reduced myocardial injury and preserved left ventricular function, as reflected by significant increase in indices of contraction (+dp/dt) and relaxation (-dp/dt). The impairment of hemodynamic and ventricular functions is the most common fatal complication of ischemic heart disease. It revealed that the extract of this herbal medicine significantly improved of the hemodynamic and ventricular functions. The present study shows that I/R control rats had lower ±dp/dt and higher LVEDP values than saline controls. It did not elucidate the mechanism responsible for this protective effect, but it is likely that HO-1 induction is at least responsible for its protective action, because HO-1 has been well documented to have anti-oxidant, anti-inflammatory and anti-apoptotic effects, which may explain why this herb reduced the production of reactive oxygen species in activated-macrophages[7]. Therefore, beneficial effect of *C. rotundus* can be extended to ischemic heart injury.

Accumulating evidence indicates that HO-1 upregulation and the subsequent increase in HO-1 activity represents an adaptive survival response to oxidative insults *in vitro* and *in vivo*. Infact, the cellular metabolism of the pro-oxidant heme into biliverdin, CO and iron is tightly regulated by HO isozymes, including constitutive HO-2 and inducible HO-1, which are highly induced by heme and oxidative stress[13]. Recently, high mobility group box-1 (HMGB1), a late cytokine in sepsis, was found to act in a specific upstream pathway to promote inflammation after cardiac I/R injury[14]. Furthermore, HO-1 inducers were found to inhibit HMGB1 release under systemic inflammatory conditions in an animal model of sepsis[15] and myocardial I/R injury[4]. Since nootkatone has been reported to reduce HMGB1 significantly under systemic inflammatory conditions in animals due to HO-1 induction[7] and reducing HMGB1 level is beneficial in myocardial I/R conditions[4], we believe that the extract of *C. rotundus* rhizomes and nootkatone protect myocardial I/R injury by induction of HO-1. However, it remains to be determined whether nootkatone reduces HMGB1 in an animal model of cardiac I/R. In order to prove the purity of nootkatone isolated from the extract, nootkatone in the extract was analyzed qualitatively and quantitatively by hyphenated LC-ESI-MS system which is used frequently to assure quality of crude plant material and products thereof[16] or quality control in the pharmaceutical industry[17]. The LC-UV technique is also versatile method for compound analysis[18], but was not available in the present study.

CONCLUSION

Summarizing, the extract of *C. rotundus* rhizomes as well as its constituent, nootkatone was found to reduce I/R injury in rat heart. We suggest that these beneficial effects are due to the anti-oxidant and anti-inflammatory effects of nootkatone, such as, HO-1 induction.

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