Int J Clin Exp Med 2016;9(9):18012-18018
www.ijcem.com /ISSN:1940-5901/IJCEM0011060

Original Article

Heme oxygenase-1 mediates the protective effect of *Rosa Laevigata Michx* on doxorubicin induced myocardial cell injury

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Received June 4, 2015; Accepted March 3, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Objective: This study is to investigate whether heme oxygenase-1 (HO-1) is involved in the protective effect of *Rosa Laevigata Michx* (RLM) on myocardial cell injury induced by doxorubicin (DOX). Methods: SD rats were randomly divided into control group, DOX group, RLM group and SnPP (tin protoporphyrin IX, a HO-1 inhibitor) group. Rats in control group received an equal volume of saline. Rats in DOX group were intraperitoneally injected with 15 mg/kg DOX. Rats in RLM group were injected with 15 mg/kg DOX intraperitoneally and given 5 g/kg RLM by gavage. Rats in SnPP group received intraperitoneal injection of 15 mg/kg DOX and 20 μmol/kg SnPP and 5 g/kg RLM by gavage. The activities of creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), HO-1 and caspase-3 were determined by colorimetry. The expression of myocardial tissue HO-1 and activation of Nrf2 were analyzed by Western Blot. Results: Compared with control group, the activities of CK-MB, LDH and caspase-3 were significantly increased ($P<0.05$) in DOX group, while HO-1 activity was only slightly increased. RLM treatment significantly inhibited the activities of CK-MB, LDH and caspase-3 in rats with DOX-induced myocardial injury, and significantly increased HO-1 activity and HO-1 expression. However, after SnPP treatment, the activities of CK, LDH and caspase-3 were significantly increased whereas HO-1 activity and expression was significantly decreased. Additionally, RLM significantly promoted Nrf2 activation. Conclusion: The protection mechanism of RLM may be through up-regulating HO-1 expression and activity.

Keywords: *Rosa Laevigata Michx*, doxorubicin, heme oxygenase-1

Introduction

Doxorubicin (DOX) is an anthracycline antibiotic used in cancer therapy [1]. Although it can inhibit many cancers such as leukemia, its accumulated adverse effects, such as irreversible cardiomyopathy and congestive heart failure, limit its use [2]. Many factors are involved in the DOX-induced cardiomyopathy, among which reactive oxygen species (ROS), calcium overload and myocardial cell apoptosis mediated by mitochondrial damage are important mechanisms [3]. In recent years, it is reported that some medicinal plants may play anti-oxidant and anti-apoptosis roles in DOX-induced myocardial injury through up-regulating the expression of heme oxygenase-1 (HO-1) [4]. Researches have shown that *Rosa Laevigata Michx* (RLM) hasstrong antioxidant ability and free radical scavenging capacity [5, 6]. The preliminary results of our research group showed that RLM had a protective effect on DOX-induced cardiotoxicity. However, the mechanism is still unclear. Therefore, this study aims to investigate the mechanism underlying the myocardial protection by RLM.

Materials and methods

Reagents

DOX was purchased from Pharmacia Co. (Batch No. 8NB002-A. Milan, Italy). Serum lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) kits were products of Stanbio Laboratory (Texas, USA). The caspase-3 colorimetric assay kit was purchased from Quantikine (R&D system, Minneapolis, MN, USA). Antibodies

against HO-1, Nrf2, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against TATA binding protein (TBP) was from ProteinTech Group (Chicago, IL, USA). RLM was purchased from Chinese Herbal Medicine Company of Shiyan (Shiyan, China). The concentration of RLM was 2.0 g/ml. Tin protoporphyrin IX (SnPP) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). PVDF membrane was from Millipore Co. (Billerica, MA, USA). The enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Animals and experimental grouping

Adult male Wistar rats were provided by the Department of Experimental Animals of Hubei University of Medicine (Hubei, China). The weight was 250 g ± 5 g. Animals were kept in SPF environment with free access to standard laboratory food and water for two weeks. The rats were randomly divided into 4 groups with 15 rats in each group. In control group (n = 15), rats received an equal volume of saline as a negative control. In DOX group (n = 15), rats were intraperitoneally injected with DOX (2.5 mg/kg, i.p.) at 48 h intervals for two consecutive weeks, and 6 times of injection were performed in total. The cumulative dose of DOX was up to 15 mg/kg. In RLM group (n = 15), rats were treated with DOX and RLM. The injection and dose of DOX was conducted as described in DOX group. RLM (5 g/kg) was given daily to rats by gavage. After two consecutive weeks, DOX injection was stopped. However, RLM treatment was continued for another 4 weeks. In SnPP group (n = 15), rats were treated with DOX, RLM and SnPP. The treatment of DOX and RLM was performed as described in DOX group and RLM group. SnPP (20 μmol/kg) was given intraperitoneally three times a week, and the duration of treatment was the same as that of RLM.

Twenty-four hours after the last RLM/SnPP treatment, rats were anesthetized with thiopentone (35 mg/kg;i.p.). Blood samples (2-3 ml each) were collected by orbital puncture and the sera were collected by centrifugation for 10 minat 3000 ×g at room temperature. The heart were rapidly dissected out, washed in ice-cold isotonic saline and homogenized in ice-cold 0.1 M potassium phosphate puffer (pH 7.4).

All efforts were made to minimize animal suffering. All animal experiments were conducted according to the ethical guidelines of Hubei University of Medicine.

Determination of the serum CK and LDH activity

Serum CK-MB and LDH activities were determined by colorimetry as described by manufacturer's instructions. The absorbance of CK-MB at 340 nm was measured using a UV-Vis Spectrophotometer (Synergy HT BioTEK, Winooski, VT, USA). Determination of LDH was performed according to the kit provided by Stanbio Laboratory, and the total LDH activity (U/L) in serum was calculated.

Determination of the myocardial HO-1 activity

The myocardial tissue homogenates were centrifuged at 18000 ×g for 10 min at 4°C. After centrifugation, the supernatant (400 μl) was mixed with the reaction mixture (containing 0.8 mmol/L NADPH, 2 mmol/L glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 2 mg of rat liver cytosol, 100 mmol/L PBS and 10 μmol/L chlorohemin). The reaction was incubated in the dark at 37°C for 1 hour and terminated with chloroform. Absorbance value was measured at the wavelength of 463 nm and 530 nm (the absorptivity of bilirubin was 40). The value of HO-1 activity was represented by the absorbance value of bilirubin, with the unit of nmol/(mg protein/h). And the relative value of HO-1 activity to the control group was calculated.

Determination of the caspase-3 activity

The myocardial tissue homogenates were centrifuged at 10000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and used to determine the caspase-3 activity. Based on the principle that caspase-3 could remove the luminescent group pNA from DEVD-pNA, caspase-3 activity was measured using colorimetric assay kit according to the manufacturer's instructions. The absorbance at 405 nm was then measured to indirectly reflect the activity of caspase-3.

Western blot analysis

The total protein and nucleoprotein of myocardial tissue was extracted, and 100 μg protein

Table 1. The effects of RLM on serum CK-MB and LDH activities (mean ± SD, n = 15)

	CK-MB (U/L)	LDH (U/L)
Control Group	194.40 ± 12.47	248.61 ± 23.08
DOX Group	390.83 ± 26.75[a]	431.72 ± 12.22[a]
RLM Group	243.79 ± 20.43[b]	267.82 ± 19.84[b]
SnPP Group	367.07 ± 17.81[a,c]	397.81 ± 25.08[a,c]

Note: [a]*P*<0.05, compared with Control Group. [b]*P*<0.05 compared with DOX Group. [c]*P*<0.05, compared withRLM group.

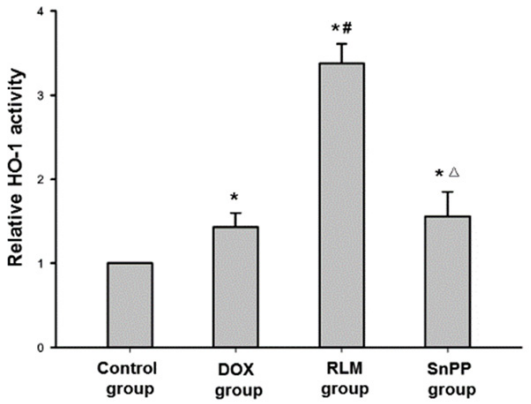

Figure 1. Analysis of HO-1 activity in rat myocardial tissue. Rat myocardial tissues were collected from control group, DOX group, RLM group and SnPP group. HO-1 activity was detected by measuring bilirubin generation. The value of HO-1 activity was represented by the absorbance value of bilirubin. Relative HO-1 activity was calculated based on values of the control group. *P<0.05, compared with control group. #P<0.01, compared with DOX group. ΔP<0.05, compared with RLM group.

was used for SDS-PAGE. The separated total protein or nucleoprotein was then transferred to PVDF membrane. The membrane was blocked with TBST containing 50 g/L non-fat milk for 1 hour at room temperature. Then primary antibodies of anti-HO-1 (dilution 1:500), anti-β-actin (dilution 1:5000), anti-Nrf2 (dilution 1:1000) and anti-TBP (dilution 1:1000) were added and incubated overnight at 4°C. β-actin and TBP proteins were used as internal controls. After washing, HRP-conjugated secondary antibody (dilution 1:10000) was added and incubated with the membrane for another 1 hour at room temperature. The membrane was developed by ECL reagent.

Statistical analysis

The SPSS 17.0 software was used for statistical analysis. Data were presented as mean ± standard deviation (SD), and analyzed using one-way ANOVA. A *P* value less than 0.05 was considered as statistically significant.

Results

RLM decreases the elevated activities of serum CK-MB and LDH induced by DOX

To determine the effect of RLM on activity of CK-MB and LDH, colorimetry assay was performed. The results were shown in **Table 1**. In comparison with the control group, serum LDH and CK-MB activities increased significantly in DOX group (*P*<0.01). However, RLM reduced activities of serum LDH and CK-MB. Compared with DOX group, activities of serum LDH and CK-MB in RLM group were significantly lower (*P*<0.05). No significant difference was found between RLM group and control group. In SnPP group, LDH and CK-MB activities were significantly higher than those of RLM group and control group (*P*<0.05), but there was no significant difference between SnPP group and DOX group (*P*>0.05). These results indicate that DOX increases activities of CK-MB and LDH and that RLM inhibits the effect of DOX by decreasing activities of CK-MB and LDH.

RLM increases the myocardial HO-1 activity

To determine HO-1 activity, colorimetry was performed. The value of HO-1 activity was represented by the absorbance value of bilirubin. Relative HO-1 activity was calculated based on values of the control group. As shown in **Figure 1**, compared with the rats of the control group, HO-1 activity increased slightly in the myocardial tissue of rats treated with DOX (*P*<0.05). However, after treatment with 5 g/kg RLM, HO-1 activity was significantly increased in RLM group compared with DOX group (*P*<0.05) and control group. After treatment with SnPP, the HO-1 activity in SnPP group was significantly lower than that of RLM group (*P*<0.05), but still significantly higher than the control group (*P*<0.05). There was no significant difference in HO-1 activity between DOX group and SnPP group. These data suggest that HO-1 activity in rat myocardial tissue is increased by RLM treatment.

RLM inhibits caspase-3 activity induced by DOX in myocardial tissue

To analyze the effect of RLM on caspase-3, capase-3 activity was detected by colorimetric

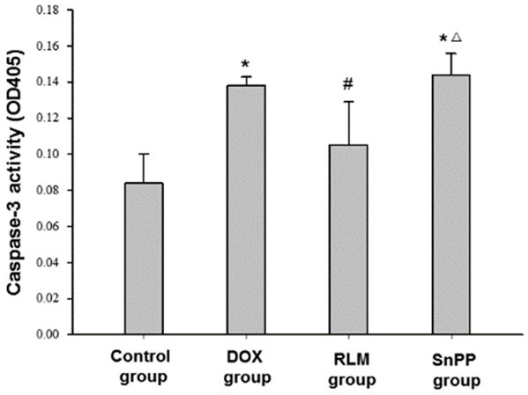

Figure 2. Analysis of caspase-3 activity in rat myocardial tissue. Rat myocardial tissues were collected from control group, DOX group, RLM group and SnPP group. Caspase-3 activity was detected with colorimetric assay kit. The absorbance at 405 nm was measured. *P<0.05, compared with control group. #P<0.01, compared with DOX group. ΔP<0.05, compared with RLM group.

assay kit. The results were shown in **Figure 2**. After treatment with DOX, the caspase-3 activity in the myocardial tissue of DOX group (0.138 ± 0.005) increased by 55%, compared with control group (0.084 ± 0.024). And the difference between control group and DOX group was significant ($P<0.05$). Caspase-3 activity in RLM group (0.105 ± 0.016) was significantly decreased than that in DOX group ($P<0.01$). However, compared with RLM group and control group, caspase-3 activity in SnPP group was significantly higher (0.144 ± 0.012) ($P<0.05$), suggesting that SnPP inhibited the effect of RLM on caspase-3. The difference between control group and RLM group or DOX group and SnPP group was not significant (P>0.05). These results showed that caspse-3 activity induced by DOX treatment was inhibited by RLM in rat myocardial tissue.

RLM enhances the expression of HO-1 inmyocardial tissue

To determine the effect of RLM on HO-1 expression in the myocardial tissue, Western Blot analysis was conducted in myocardial tissues of control group, DOX group, RLM group and SnPP group. β-actin was used as an internal control. Representative Western Blot results were shown in **Figure 3A**. Compared with control group, the HO-1 expression level increased slightly in myocardial tissues of rats treated with DOX. Meanwhile after treatment with RLM, the HO-1 expression was further enhanced in RLM group. Additionally, SnPP treatment reduced the expression of HO-1 in SnPP group. Thus, this data indicate that RLM increased the HO-1 expression, which was consistent with its effect on HO-1 activity.

RLM promotes Nrf2 translocation from the cytoplasm to the nucleus

To analyze whether RLM affects the translocation of Nrf2, we detected expression of Nrf2 in cell nuclei of myocardial tissues by Western Blot analysis. TBP was used as an internal control. Representative Western Blot results were shown in **Figure 3B**. In comparison to rat myocardial tissues from control group, Nrf2 content in the nuclei of the DOX treated rat myocardial tissue was not obviously increased. However, after treatment with RLM, the Nrf2 level in nucleus was obviously increased in RLM group, indicating that RLM could promote Nrf2 translocation from the cytoplasm to the nucleus. These data suggest that RLM promotes activation of the transcription factor Nrf2.

Discussion

DOX is a DNA-acting anthracycline antibiotic which can intercalate into DNA, and is widely used in clinic. Acute side effects of DOX include nausea, vomiting and arrhythmias [7]. During long-term use, DOX can interact with iron and generate a large number of reactive oxygen species during the process of mitochondrial oxidative phosphorylation, which can damage myocardial and cause myofibrillar damage or apoptosis [8, 9]. HO-1 is the rate-limiting enzyme that catalyzes the degradation of heme to CO, ferrous ion and bilirubin, and plays important roles in inflammatory and oxidative damage in the body [4]. This study showed that after treating rats with DOX-induced myocardial injury with RLM, the HO-1 activity and expression were significantly increased. HO-1 gene promoter region contains a binding site of the transcription factor Nrf2. This study also showed that RLM could induce the translocation of Nrf2 from the cytoplasm to the nucleus, thereby activating the Nrf2 and inducing the HO-1 expression.

Nrf2 is an important acquired protective nuclear transcription factor of the body. Non-activated Nrf2 binds to the inhibitor keap1 in

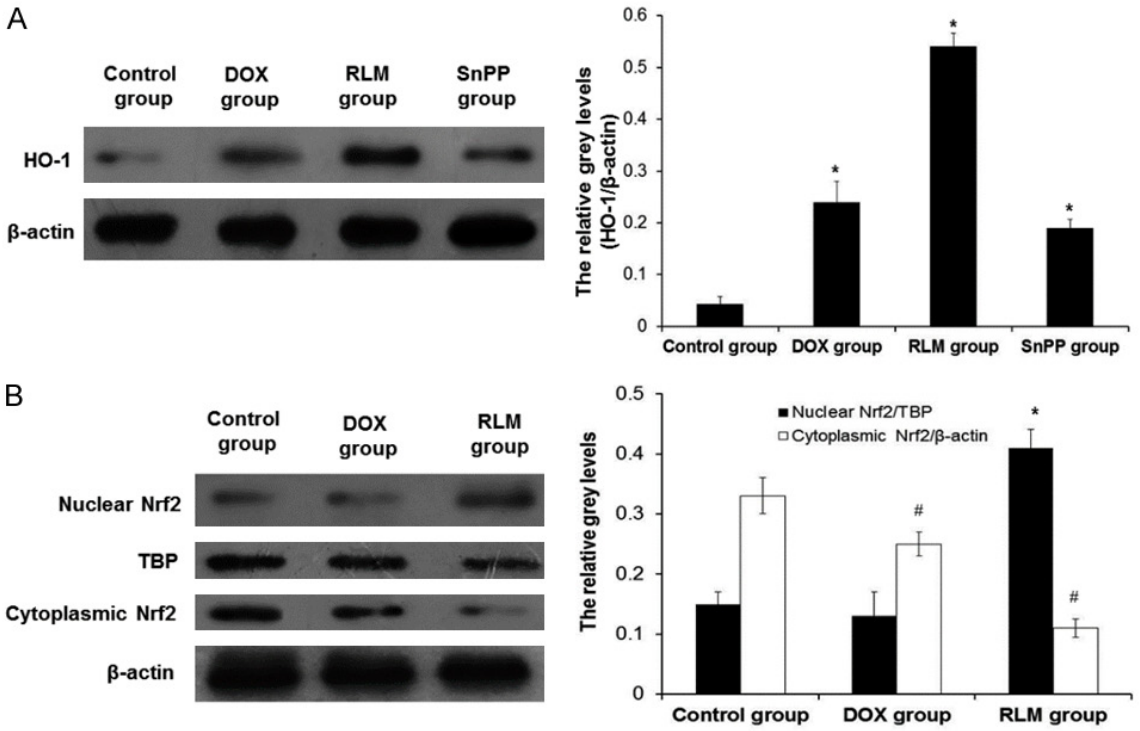

Figure 3. Analysis of HO-1 and Nrf2 expressions in rat myocardial tissues. Expression levels of HO-1 and Nrf2 were detected by Western Blot analysis. A. Rat myocardial tissues were collected from control group, DOX group, RLM group and SnPP group. Representative Western Blot results of HO-1 expressions of each group were presented and β-actin was used as an internal control. The relative grey levels of the bands were presented in the right panel. B. Rat myocardial tissues were collected from control group, DOX group and RLM group. Representative Western Blot results of Nrf2 expression were presented. β-actin was used as acytoplasmic internal control and TBP was used as a nuclear internal control. The relative grey levels of the bands were presented in the right panel. *P<0.05, compared with control group for nuclear Nrf2 level; #P<0.05, compared with control group for cytoplasmic Nrf2 level.

the cytoplasm [10, 11]. When cells are subjected to exogenous stimuli, Nrf2 separates from keap1, transfers to the nucleus, and thus binds with the antioxidant response elements of stress-induced genes, including the antioxidant response element in the HO-1 promoter region [5]. It was found in this study that the Nrf2 translocation increased after RLM treatment, which was consistent with the changes in HO-1 protein expression and activity in the cardiac tissue. These results indicate that RLM-induced HO-1 expression may be due to the translocation and activation of Nrf2. The metabolites of HO-1, such as CO and biliverdin, play the roles of antioxidant and anti-inflammatory in a variety of lung diseases including acute lung injury. Their effects include inhibition of the formation of proinflammatory cytokines, chemokines and ROS [6]. To further evaluate the role of HO-1 in the protective effect of the RLM, rats were further treated with SnPP (a HO-1 inhibitor). The myocardial injury indicators of CK-MB and LDH were detected. The results showed that the serum LDH and CK-MB activities in DOX group were significantly higher than those in control group, suggesting that DOX induced myocardial injury in rats. However, serum LDH and CK-MB activities in RLM group were significantly reduced compared with those in DOX group. This result indicates that RLM alleviated the myocardial injury induced by DOX in rats. Furthermore, the protective effect of RLM was significantly reversed after treatment with SnPP. This further verified that RLM exerted protective effects on myocardial cells through upregulating HO-1 protein expression and thus increasing HO-1 activity.

There are many mechanisms underlying DOX-induced myocardial cell injury, including ions and radicals hypothesis, metabolic mechanism and apoptosis mechanism [12, 13]. Myocardial cells are terminally differentiated cells. Various

anticancer drugs can cause the necrosis or apoptosis of myocardial cells, thus leading to loss of myocardial cells and inadequate systolic function of the heart [14, 15]. Studies have shown that DOX can cause myocardial apoptosis by endogenous and exogenous mechanisms, and finally activate the downstream factors of caspase-3, caspase-6 and caspase-7 [16, 17]. Once the caspases are activated, the cell enters apoptotic execution phase. It was found in this study, RLM inhibited the DOX-induced caspase-3 activation. However, after treatment with the HO-1 inhibitor SnPP, the caspase-3 activity increased again. This data suggest that HO-1 could impair the pro-apoptotic effect of DOX in a certain extent, and thus inhibit the DOX-induced cardiomyocyte apoptosis, necrosis and myocardial fibrosis.

In summary, data of this study showed that RLM promoted the nuclear translocation of Nrf2, thereby induced upregulation of HO-1 expression in myocardial tissue and enhanced-HO-1 activity, and further inhibited DOX-caused myocardial apoptosis.

Acknowledgements

This work was supported by Shiyan City key scientific and technological projects (2009S48).

Disclosure of conflict of interest

None.

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