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Ethyl Acetate Extract of *Radix Cynanchi Auriculati* Exerts Antioxidant Effects on LPS-induced RAW264.7 Cells by Regulating MAPKs/Nrf2/HO-1 Pathways

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Abstract: Extracts of Radix Cynanchi Auriculati (RCA) have multiple effects. However, it remains unclear whether RCA extracts can exhibit antioxidant activity by regulating MAPKs/Nrf2/HO-1 pathways. In this study, we evaluated the antioxidant activity of ethyl acetate extract of RCA (RCAEA) via LPS-induced RAW264.7 cells, and investigated the potential mechanism of its protective effect against oxidative damage on macrophages. The results showed that RCAEA activating MAPKs/Nrf2/HO-1 pathways. In conclusion, the aim of this study was to describe the molecular mechanisms responsible for the RCAEA mediating the inhibitory effects of oxidative stress in RAW264.7 cells.

Keywords: RCAEA, Oxidative stress, MAPKs/Nrf2/HO-1 pathways.

1. Introduction

Similarly, mitogen-activated protein kinases (MAPKs) are a family of kinases consisting of extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK (p38), which affect the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway (Cargnello and Roux, 2011). As the level of factors increases, reactive oxygen species (ROS) also accumulate, and they act as two distinct processes that are interrelated and together influence the oxidative stress process (Tang, Diao, Shu, Li and Xiong, 2019). There is a growing interest in health-conscious and non-toxic natural herbs for the prevention and treatment of diseases related to oxidative stress, as an alternative therapy (Yang, Wang, Xu, Ren, Tang, Gong, Lin, Fang and Su, 2022, Yatoo, Gopalakrishnan, Saxena, Parray, Tufani, Chakraborty, Tiwari, Dhama and Iqbal, 2018). As an important bioactive substance, the root extract of RCA has been shown to have neuroprotective, antitumor and skin protective effects (Wang, Cai, Zhao, Tian, Kong, Sun, Liu, Chen, An, Wang, Liu, Wu and Zhou, 2021). Recent studies have shown that C21-steroidal glycosides, obtained by ethanol extraction followed by ethyl acetate (EA) extraction, have antioxidant effects (Wu, Wang, Meng, Wang, Li, Qian, Wei, Shu, Ding, Wang and Peng, 2018). However, to date there are still some gaps in the reports on the EA extract of RCA (RCAEA) as an antioxidant or adjuvant and it is time to focus more on the underlying mechanisms of its beneficial biological activity and potential synergistic and antagonistic effects.

The purpose of this project was to identify the possible protective role of RCAEA on LPS-induced RAW264.7 cells by detecting oxidative stress indicators, and further explore its underlying molecular mechanism. These results may shed insights into the potential protective mechanism of ethanol extract from RCAEA.

2. Materials and Methods

2.1. Materials and reagents

RCA was harvested from Binhai County, Jiangsu Province, China, in December 2020 and stored in a shaded place waiting for use. Analytical grade ethanol, petroleum ether, methylene chloride, ethyl acetate and n-butanol purchased from Energy Chemistry (Shanghai, China). Trypsin, Arc-Bis, Trisma Base and Glycine were purchased from Biosharp (Guangzhou, China). RIPA Lysate and BCA Test Kit was purchased from KGI Biotechnology, co, Ltd (Jiangsu, China). All antibodies were from Beyotime (Shanghai, China), and ECL Western Blotting Substrate was from Proteintech (Chicago, USA).

2.2. Sample preparation

The fresh RCA samples (100 g) were extracted with 1000mL of 70% ethanol. Reflux extraction was performed three times (80 ◦C; 2, 1.5, 60 min, respectively). The solvent was then filtered and concentrated at 40◦C to obtain the ethanol extract (EE). EE was dissolved in distilled water and extracted successively with petroleum ether, dichloromethane, ethyl acetate and n-butanol. For further separation, the rotary evaporator was used for decompression and concentration, and the dried extracts were treated with a freeze dryer. Finally, 0.65 g of petroleum ether extract (PEE), 0.97 g of dichloromethane extract (CE), 0.93 g of ethyl acetate extract (EAE), 1.05 g of n-butanol extract (NBE), and 7.87 g of water extract (WE) were obtained. For testing, the extract powder was dissolved in dimethyl sulfoxide (DMSO) and further diluted in cell culture medium to obtain the final concentration for bioassay purposes.

2.3. Cell culture and cell sensitivity assays

RAW264.7 macrophage cells were provided from Zhong Qiao Xin Zhou Biotechnology, co, Ltd. (Shanghai, China). The macrophages were cultured in DMEM medium (containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C a humidified atmosphere of 5% CO2. The reserve solution of each extract was prepared before the

experiment. Chromatographic dimethyl sulfoxide (DMSO) was used to dissolve the lyophilized extract of RCA, and the drug was diluted to each concentration by gradient dilution method. Three auxiliary Wells were set for each concentration, and 10 μL of the prepared drug was added to each well. Ensure that the drug concentration in each well of the final 96-well plate is 25, 50, 100, 200, 400 and 800 μg/mL, LPS (200 ng/mL) was added for 24 h (Ren, Su, Li, Cai, Zhang, Zhai, Li, Wu and Hu, 2020). Then, 10μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well, 4 h later, the vacuum pump was attached to the wall of the well plate, and the cell supernatant in each well was slowly absorbed, then 100μL DMSO was added to each well. After the crystallization was completely dissolved, the absorbance value of each well was read at 570 nm and 630 nm using a microplate reader. The cell survival rate was determined within 24 h.

2.4. ROS level detection

The content of reactive oxygen species (ROS) in LPS-induced RAW264.7 macrophages was determined according to the instructions of ROS Detection Kit. After pretreatment with RCAEA, RAW264.7 cells were then co-treated with LPS (200 ng/mL) for 24h, and cleaned with fresh serum-free medium for 2-3 times. In the positive control group, 50 μL Rosup was added (the concentration was prepared according to the instruction of ROS Detection Kit), and culture was continued for 30 min. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) diluted with fresh serum-free medium was added into each well of experimental groups. After processing, ROS were quantified according to the wavelength range shown in the kit (Ren, Yuan, Wang, Zhang, Wang, Li, Zhang and Hu, 2019).

2.5. Immunofluorescence assay

The 24-well plates containing RAW264.7 macrophages were taken out from the incubator, and 4% paraformaldehyde was added to each well at room temperature to fix the macrophages for 25 min. After rinsed by PBS, the macrophages were permeated with 0.1% Triton 100 for 20 min, rinsed again, and the cells were sealed with 1% BSA for 30 min. Incubation was carried out overnight with appropriate polyclonal antibody (1:100) in blocking solution at 4 ℃. On the next day, and then a secondary fluorescein isothiocyanate (FITC) conjugated IgG antibody to observe the cellular localization of Nrf2. Finally, the cells stained with Hoechst 33258 were observed and analyzed by a fluorescence inverted microscope (FSX100, OLYMPUS, Japan).

2.6. Western blotting

The extraction method of protein was compared with previous reports, with slight modifications (Pap, Pandur, Jánosa, Sipos, Agócs and Deli, 2021). First, RAW264.7 cells in 6-well plates were collected and lysed with WIP lysate for 30 min. Protein was quantified with a 96-well plate in advance, and absorbance was measured at 562 nm after dilution with BSA protein standard. Protein concentration could be obtained according to the drawn standard curve. The cell extracts were modified with 6%-15% SDS-PAGE, and the isolated proteins were transferred to PVDF membrane by electrophoresis. The imprinted PVDF membrane was cut and incubated with primary antibody at 4 ◦C at night. Finally, the western blotting was quantified using software Image J (Bethesda, Maryland, USA).

2.7. Data analysis

GraphPad Prism 5 Software (Version 8.0, GraphPad Software, Inc. San Diego, USA) was used for statistical processing of all data, and all data were expressed as mean ±SD of independent experiments. In addition, P-value ≤0.05 or less is considered statistically significant in a single comparative statistical analysis using the T-test method. All measurements were performed in triplicates.

3. Results

3.1. Cytotoxic effect of RCAEA on LPS-induced RAW264.7 cells

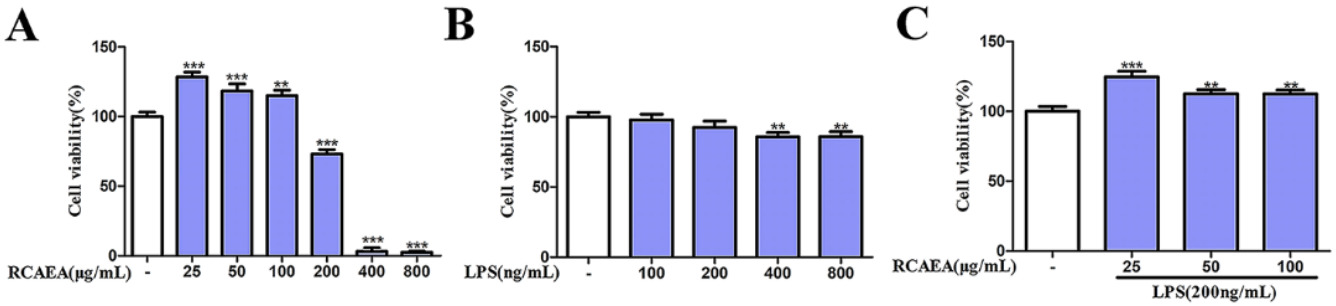

Figure 1. Effect of RCAEA on RAW264.7 macrophages survival rate. (A) Effect of RCAEA on the toxicity of cells. (B) Effect of LPS on the toxicity of cells. (C) Effect of RCAEA on the toxicity of LPS-induced RAW 264.7 cells. Values = mean ± SD, n = 3 (**P < 0.01, ***P < 0.001, compared to the LPS-treated group).

Firstly, we examined the effects of different concentrations of RCAEA (25-800 μg/mL) and different concentrations of LPS (100-800 ng/mL) on cell viability, respectively. The results showed that RCAEA showed low toxicity to cells up to a concentration of ≤100 μg/mL (Fig.1A), and LPS was almost non-toxic to cells at concentrations ≤200 ng/mL (Fig.1B). Meanwhile, to avoid combined toxicity of both, we also investigated the cytotoxicity of co-cultures using LPS (200 ng/mL) and RCAEA (25, 50, 100 μg/mL). The results showed that both had almost no inhibitory effect on cell proliferation, even at the highest doses (Fig. 1C). Therefore, the concentration of RCAEA at 25, 50, 100 μg/mL was selected for further study.

3.2. RCAEA clears ROS by regulating Nrf2/HO-1 signaling pathway

Under oxidative stress conditions, intracellular ROS levels

are elevated. At this time, Nrf2, which is originally present in the cytoplasm, is increased in the nucleus and exerts a regulatory effect on oxidative stress by enhancing the transcription of anti-oxidative stress proteins such as HO-1 (Bellezza, Giambanco, Minelli and Donato, 2018, Zhang, Zhang, Chen, Hu, Wang, Jin, Zhang, Wang, Wang, Kang, Li, Li, Pan, Huang and Kong, 2015). As shown in Figure 2A, ROS levels were significantly increased in LSP-induced cells, and RCAEA treatment was effective in scavenging intracellular ROS levels in a dose-dependent manner, suggesting that RCAEA may have antioxidant effects. To investigate whether the ROS scavenging ability of RCAEA is mediated by Nrf2, we examined the location and gene expression of the nuclear factor Nrf2 in RAW264.7 macrophages. The results suggest that with the increase of RCAEA concentration, Nrf2 was gradually transferred from the concentrated cytoplasm to the nucleus (Fig.2B), and the mRNA expressions of Nrf2, HO-1 and NQO1 were significantly increased in RAW264.7 macrophages, while Keap1 mRNA was inhibited to a certain extent (Fig.2C). The results of the western blot assay showed the same trend (Fig.2D). In addition, RCAEA treatment increased HO-1 expression and inhibited Keap1 expression in macrophages (Fig.2E, F).

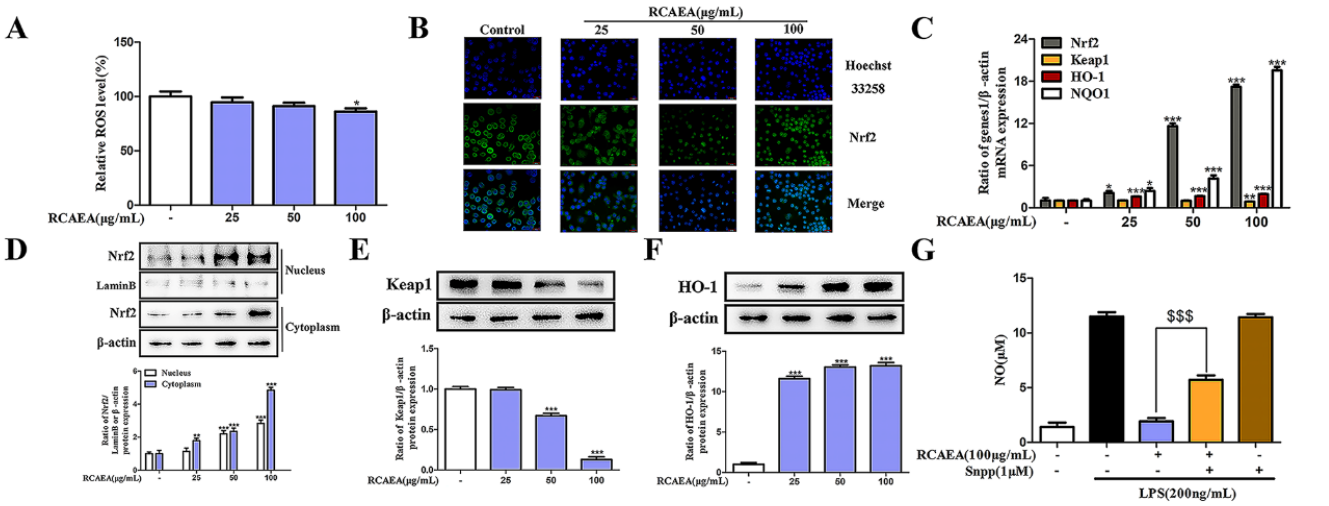

Figure 2. Inhibition effect of RCAEA on Nrf2/HO-1 signaling pathway in LPS-induced RAW264.7 cells. (A) Effect of RCAEA on ROS levels. (B) Nrf2 localization images of RAW264.7 whole cells scanned after RCAEA treatment. (C) Effects of RCAEA on the expression of four key genes in Nrf2/HO-1 signaling pathway in cells. (D) Effects of RCAEA on Nrf2 protein expression in and outside the nucleus was demonstrated by Western blot. (E and F) Effects of RCAEA treatment on Keap1 and HO-1 protein expression after 4h, total protein of cells was extracted by Western blot. (G) The influence of Snpp on the change of intracellular NO production under RCAEA treatment was determined by Griess Kit. The blank control group was given no RCAEA and inhibitor, and the positive control group was given inhibitor only. LaminB and β-actin were used as the internal control for normalization. Values = mean ± SD, n = 3 (*P <0.05, **P < 0.01, ***P < 0.001 compared to the control group and $$$P < 0.001 compared to the RCAEA plus LPS-treated group).

HO-1 is a downstream gene of Nrf2, which is a potent antioxidant target (Ren, Su, Li, Cai, Zhang, Zhai, Li, Wu and Hu, 2020). To verify whether the response is involved in RCAEA-mediated activation of the Nrf2/HO-1 signaling pathway, we used Snpp, which is an HO-1 inhibitor, to treat RAW264.7 cells and assay the levels of NO release. The results showed that RCAEA significantly inhibited the release of NO induced by LPS, while the addition of HO-1 inhibitor resulted in an increase in NO release (Fig.2G).

3.3. RCAEA improves antioxidant response by regulating MAPKs/Nrf2/HO-1 signaling pathway

Phosphorylation of protein kinase B (Akt) and MAPKs is critical for NF-κB activation and subsequent release of mediators (Zhao, Ma, Cai and Gong, 2019). It is also engaged in modulation of the Nrf2/HO-1 signaling pathway (Bryan, Olayanju, Goldring and Park, 2013). Therefore, we treated RAW264.7 macrophages with RCAEA for 15, 30 and 60 min, respectively, to detect the expression of Akt, ERK, JNK and p38. The results showed that the phosphorylation level of Akt in RCAEA-treated RAW264.7 macrophages was significantly increased and reached a peak at 15 min. In addition, the phosphorylation levels of ERK, P38 and JNK were also significantly increased, indicating that RCAEA can activate MAPKs signaling pathway through phosphorylation of Akt (Fig.3A). To confirm whether the biological functions of RCAEA via activates Nrf2/HO-1 signaling pathway through phosphorylation of MAPKs, the ERK specific inhibitor (PD98059), JNK specific inhibitor (SP600125) and p38 specific inhibitor (SB203580) were used in RAW264.7 macrophages. As shown in Figure 3B-C, all three inhibitors reduced Nrf2 and HO-1 protein levels after RCAEA treatment, with the inhibition being most pronounced after co-treatment of the p38-specific inhibitor (SB203580) with RCAEA, suggesting that the antioxidant effects of RCAEA may be largely dependent on the Nrf2/HO-1 signaling pathway mediated by p38 phosphorylation. These results suggested that RCAEA could reduce improves some responses by regulating MAPKs/Nrf2/HO-1 signaling pathway.

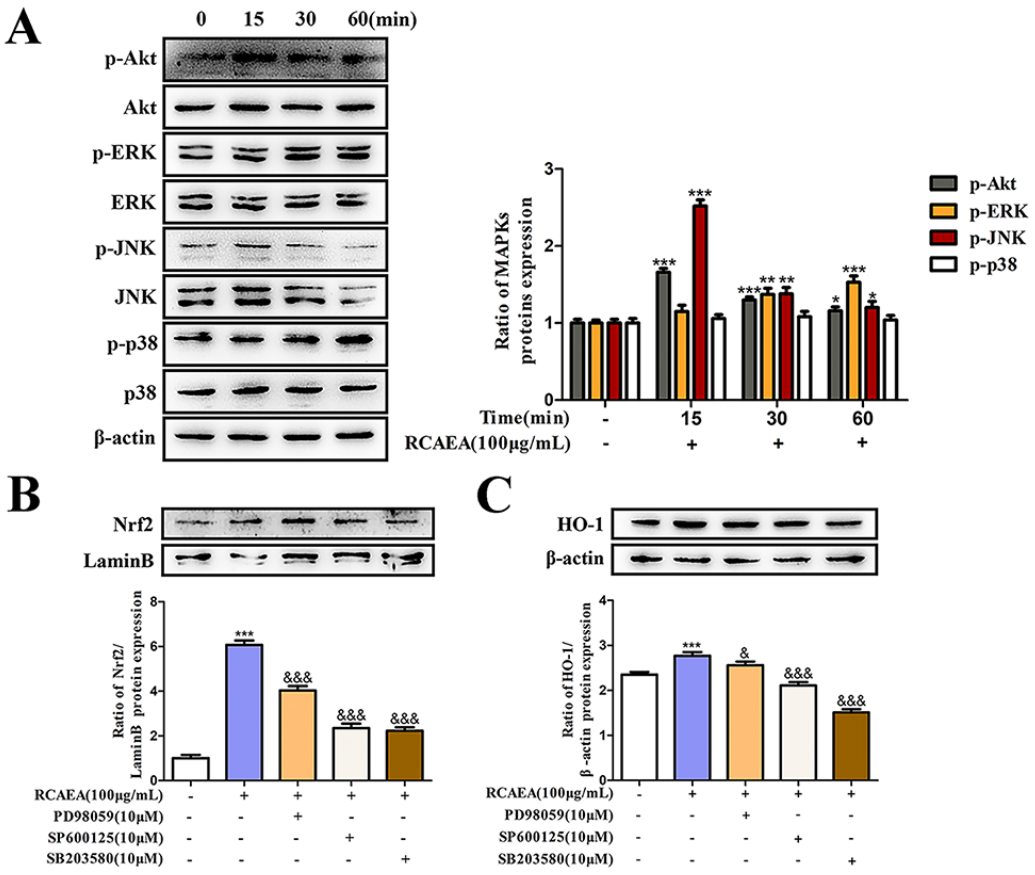

Figure 3. Effect of RCAEA on the regulation of MAPKs/Nrf2/HO-1 signaling pathway in LPS-induced RAW264.7 cells. (A) Comparison of p-Akt, P-ERK, P-JNK and P-P38 levels between the treatment group and the control group. (B and C) Expression of Nrf2 and HO-1 in cells after pretreatment with MAPKs inhibitors and RCAEA. LaminB and β-actin were used as internal controls for normalization. Values = mean ± SD, n = 3 (*P <0.05, **P < 0.01, ***P < 0.001 compared to the control group and &P < 0.05, &&&P < 0.001 compared to the RCAEA-treated group).

4. Discussion

It is a well-known thing that ROS can also act as second messengers, regulating multiple signal kinase cascades and activating related transcription factors (Qi, Feng, Li, Qi and Zhang, 2017). Under normal circumstances, the ROS production and clearance system are in a dynamic balance, but in pathological conditions, the dynamic balance of oxygen free radical production and clearance is damaged, and when the ROS production rate is greater than the clearance rate, the relative accumulation of ROS will be caused (Li, Liu, Dai, Jiang and Zhao, 2021). Therefore, ROS scavenger reagents are required to maintain healthy ROS levels. In our study, we found that ROS levels were significantly increased in LPS-induced cells. While RCAEA treatment significantly clarified the excess intracellular ROS, indicating that RCAEA has antioxidant capacity. We investigated the effect of RCAEA on the Nrf2/HO-1 signaling pathway. In conclusion, the antioxidant activity of RCAEA may be induced by nuclear transfer of Nrf2 and increased HO-1 expression.

Protein kinase pathways associated with the cellular stress response are closely related to the regulation of HO-1, including MAPKs. MAPKs signaling pathway is also inextricably tied to the progression of inflammation, and the main mechanism is to mediate signaling into the nucleus by receiving extracellular stimuli (Chen, Jia, Li, Han, Li, Zhang, Li, Yuan and Wu, 2020). JNK and p38, as major kinases in MAPKs, are activated after a series of phosphorylation events (Johnson and Lapadat, 2002). In the current study, after the phosphorylation of MAPKs under RCAEA protection was confirmed, we also studied the relationship between MAPKs and downstream pathways related to oxidative stress. By using inhibitors of JNK, ERK and p53, we found that the expression of Nrf2 and HO-1 was inhibited after RCAEA treatment, in which p38 inhibitors were more effective. This evidence not only suggests that the MAPKs signaling pathway may be involved in the RCAEA-mediated regulation of Nrf2 and HO-1 expression, but also indicates that RCAEA is more likely to exert its ultimate antioxidant effects by regulating p38. Taken together, this evidence suggests that RCAEA may achieve its antioxidant capacity by regulating the MAPKs/Nrf2/HO-1 pathway in RAW264.7 macrophages.

5. Conclusion

In summary, we successfully extracted effective antioxidant active components from RCA and validated in RAW264.7 cells. The results showed that RCAEA exerted strong oxidative stress protective effects on LPS-induced RAW264.7 cells. Further studies revealed that the protective effects of RCAEA on macrophages were mainly via induction of MAPKs phosphorylation and activation of Nrf2/HO-1 signaling pathway.

6. Ethics Statement

This manuscript did not include any human subjects and animal experiments.

7. Credit Author Statement

The experiments for this work were designed by Prof. Ren. The experiments were done by Ya Su and Lili Zeng. Su Ya, Jichao Zhou and XingYi analyzed the data, edited the graphs and tables, and wrote the manuscript. Professor Jie Ren reviewed the manuscript. Other authors provided reagents.

8. Conflict of Interest

The authors declared that no conflict of interest.

Acknowledgment

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