

RESVERATROL SUPPRESSED LPS-INDUCED COX-2 VIA miR-146a-5p INHIBITION IN Raw246.7 CELLS

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Abstract

Trans-resveratrol (Res) is a well-known natural stilbene frequently found in grapes which have been reported to possess antioxidant, anti-cancer activities and inhibited COX-2 expression. MicroRNAs (miRNAs) are short endogenous non-coding RNAs involved in the regulation of mRNA stability and protein synthesis. In our research, resveratrol isolated from *Vitis heyneana* Roem. & Schult *Vitis heyneana* was observed to suppress lipopolysaccharides (LPS)-induced COX-2 expression in Raw264.7 cells in a dose dependent manner. Using qPCR it was revealed that LPS induced the expression of miR-25, miR-125a, miR-125b, miR-146a-5p, miR-146a-3p and miR-455. However, we only observed miR-146a-5p expression significantly decreased in resveratrol compared to untreated-control group. In addition, resveratrol abrogated the effect of miR-146a-5p mimic induced-COX-2 expression in Raw264.7 cells. Taken together, this study demonstrated for the first time the involvement of miR-146a-5p in resveratrol inhibited LPS-induced COX-2 expression in Raw264.7 cells.

Rezumat

Trans-resveratrolul (Res) este un stilbenoid natural, întâlnit frecvent în struguri, care a demonstrat proprietăți antioxidante și anticanceroase prin inhibiția expresiei COX-2. MicroARN (*miRNAs*) sunt molecule endogene de ARN, implicate în reglarea stabilității ARNm și a sintezei proteice. În acest studiu, resveratrolul izolat din *Vitis heyneana* Roem. & Schult *Vitis heyneana* a inhibat, doză dependent, expresia COX-2 indusă de lipopolizaharide (LPS), în celulele Raw264.7. Prin tehnica qPCR a fost demonstrat că LPS induc expresia miR-25, miR-125a, miR-125b, miR-146a-5p, miR-146a-3p și miR-455. Cu toate acestea, s-a observat că resveratrolul a produs o scădere semnificativă a expresiei miR-146a-5p față de martor. În plus resveratrolul a anulat efectul COX-2 indusă de miR-146a-5p în celulele Raw264.7. Prin urmare s-a dovedit implicarea miR-146a-5p în inhibiția mediată de resveratrol a COX-2 indusă de LPS în celulele Raw264.7.

Keywords: Resveratrol, COX-2, miR-146a-5p, Raw264.7 cells; *Vitis heyneana* Roem. & Schult

Introduction

Cyclooxygenase (COX) is an enzyme that is responsible for the formation of prostanoids, including prostaglandins, prostacyclin and thromboxane [1]. COX-1 is an enzyme that acts in order to speed up the production of certain chemical messengers, called prostaglandins, and is present in most tissues, whereas COX-2 is an inducible form, primarily present at sites of inflammation and COX-2 is increased by oncogenes, growth factors, cytokines or endotoxin [2]. Overexpression of COX-2 is responsible for the prostaglandin E2 production (PGE2), an inflammatory mediator participating in many biological processes, including inflammation, pain, carcinogenesis, angiogenesis and cancer [3, 4].

The discovery of the short endogenous noncoding RNAs called microRNAs (miRNAs) suggested a new regulatory mechanism for cellular signalling and protein expression. miRNAs was first discovered in 1993, however, there are numerous studies demonstrated the role of miRNA play a fundamental role in a number of human disease states, including cancer, diabetes, cardiovascular diseases or inflammatory diseases [5-7]. Resveratrol, a well-known natural polyphenol found in plants and fruits such as grapes, berries [8] has antioxidant, anti-aging, anti-cancer, anti-atherosclerosis, anti-inflammatory activity [9]. According to Pubmed database, the effect of resveratrol on COX-2 expression was assessed in 183 papers. However, there is only one paper that reported that resveratrol

decreased glioma cell growth involved some miRNAs (miR-21, miR-30a-5p and miR-19) on rat.

Vitis heyneana, a species of wild-climbing grape in Vietnam has been used for inflammatory diseases. In the present study, we isolated resveratrol from *Vitis heyneana* and examined the effect of resveratrol on LPS-induced COX-2 expression in Raw264.7 cells. Furthermore, we first reported miR-146a-5p mimic induced COX-2 expression and resveratrol suppressed miR-146a expression in Raw264.7 cells. Moreover, resveratrol abrogated the expression of COX-2 induced by miR-146a-5p mimic. Thus, we first demonstrated the role of miR-146a in COX-2 expression response to resveratrol in Raw264.7 cells.

Materials and Methods

Plant Material

The whole aerial parts of plant samples (6.0 kg) were collected from Si Ma Cai-Lao Cai province, Vietnam, and botanically identified as *Vitis heyneana* Planch. (*Vitaceae*) by a specialist. A voucher specimen (TL07) has been deposited at the Herbarium of Institute of Ecology and Biological Resources, Vietnam.

Extraction

The dried materials of *Vitis heyneana* (5.0 kg) were extracted under heat with 96% ethanol (36 L × 3 times × 3 hr). The combined 96% ethanol extracts were concentrated under vacuum to yield a dry residue of 234.5 g. This crude extract was then suspended in H₂O (2 L) and partitioned successively with *n*-hexane (3 × 1.5 L) (Hx), ethyl acetate (3 × 1.5 L) (EtOAc), and residue (VHW). Pooled extracts were combined and evaporated under vacuum to obtain extract residues including 26.05 g of VHH from Hx (account for 11.3%), 129.67 g of VHET from EtOAc (account for 55.4%), and 50.4 g of VHW from water layer (account for 21.5%).

Isolation

The EtOAc fraction (34.0 g) was subjected to chromatography over a silica gel column (5 × 20 cm; 63 - 200 μm particle size) and eluted with DCM-MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 1:100, each 1.0 L) to yield 7 fractions VHE1-VHE7 followed by checking TLC profiles. The fraction VHE4 (1.5 g) was continuously applied to a silica gel column, eluted with dichloromethane (DCM): MeOH (50:1, 20:1, 10:1, 5:1) to give 5 sub-fractions VHE4.1-VHE4.5. The fraction VHE4.2 (200 mg) was further purified using a Sephadex LH-20 column (3 × 30 cm) using MeOH as an eluting solvent and then to obtain compound VHR (18 mg, resveratrol). VH: a pale powder; M.p. 254 - 255°C; ¹H-NMR (500 MHz, CD₃OD) δ_H: 6.47 (2H, d, 2 Hz, H-2, H-6), 6.18 (1H, t, H-4), 7.36 (2H, d, 8.5 Hz, H-2', H-6'), 6.79 (1H, d, 8.5 Hz, H-3'), 6.78 (1H, d, 8.5 Hz, H-5'), 6.83 (1H, d, 16.3 Hz, H-a), 6.96 (1H, d, 16.3 Hz, H-b); ¹³C-NMR (125 MHz, CD₃OD) δ_C: 141.3 (C-1),

105.8 (C-2, C-6), 159.6 (C-3, C-5), 102.7 (C-4), 130.4 (C-1'), 128.8 (C-2', C-6'), 116.5 (C-3', C-5'), 158.3 (C-4'), 127.0 (C-a), 129.4 (C-b). ESI-MS *m/z* 227 [M-H]⁻, calculated *m/z* for C₁₄H₁₂O₃ is 228. [10]. The purity of the compound VH were greater than 95%, as estimated using an HPLC-DAD method.

Materials

DMEM, trypsin, foetal bovine serum, were supplied by Gibco BRL (Grand Island, NY). COX-2 (1:1000, cat: 610204), and HSP90 (1:1000, cat: 610418) were obtained from BD Biosciences. Secondary mouse or rabbit HRP-conjugated antibodies (1:5000 or 1:10000, Cell Signalling, #7074, #7076), LPS (cat: L4391) were purchased from Sigma Aldrich. Control mimic and miR-146a-5p miScript miRNA mimic were purchased from Qiagen.

Cell culture and transfection

Raw 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO₂ - 95% air. For all experiments, cells were grown to 80% - 90% confluency and subjected to no more than 20 cell passages. Cells were transfected with control mimic or mimic at a concentration of 20 nM using Oligofectamine transfection reagent (Life Technologies) according to the manufacturer's instructions.

Western blot analysis

After treatment, cells were collected and washed with cold phosphate-buffered saline (PBS). The collected cells were then lysed on ice for 30 min in 100 μL lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP40 (Nonidet P-40)] and centrifuged at 12,000 rpm for 30 min. Supernatants were collected from the lysates and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with primary antibodies or mouse monoclonal β-actin antibodies. The membranes were further incubated with secondary anti-mouse or anti-rabbit antibodies. Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection West Femto Kit (Thermo Scientific, Catalogue: #34096).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using miRNeasy mini kit (Qiagen, #217004) according to the manufacturer's instructions. The expression of miRNA and mRNA was analysed by real-time qPCR (StepOnePlus qPCR cycler, Applied Biosystems) using miScript SYBR Green RT-PCR Kit Qiagen, (cat: #218073), miScript primer assays (Qiagen): Mm_miR-125a, Mm_miR-146a-5p, Mm_miR_455, Mm_miR-155, Mm_miR-

146a-3p, Mm_miR-25, Snord 95 and QuantiFast SYBR Green RT-PCR Kit Qiagen, #204156) and QuantiTect primer assays (Qiagen): COX-2 (Ptg2), Mm_GADPH.

Statistics

Values are presented as mean \pm S.E. unless otherwise stated. p-values were calculated by Student's t-test or one-way analysis of variance followed by Bonferroni post-hoc testing using GraphPad Prism 5 (GraphPad Software Inc.). $p < 0.05$ was considered statistically significant. Data are expressed as mean \pm S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results and Discussion

COX-2 overexpression has been observed in pain, immunity, cancer or inflammation development [1, 2]. In order to screen drug candidates that suppress COX-2 expression in Raw264.7 cells, we initially incubated cells with or without LPS and stimulated with extracts from *Vitis heyneana* at 50 $\mu\text{g}/\text{mL}$ (hexan,

buthanol, ethylacetate and methanol extraction) and the main component isolated from *Vitis heyneana* resveratrol at 30 μM . Figure 1A showed resveratrol most effectively decreased LPS-induced COX-2 expression by qPCR. To confirm this result, cells were incubated with LPS and resveratrol for 18 hr and then cells were collected for qPCR. As expected, resveratrol, in a dose-dependent manner, suppressed LPS-induced COX-2 expression in Raw264.7 cells (Figure 1B). Recently, some studies demonstrated that COX-2 expression can be regulated by miRNAs. Moreover, Monk *et al.* showed LPS induced miR-455, miR-125, miR-146 and miR-155 in macrophages [11]. Also, miR-25 was reported in the regulation of COX-2 [12]. To confirm this, we treated Raw264.7 cells with LPS (5 $\mu\text{g}/\text{mL}$) for 24 hr and then harvested samples for qPCR. As shown as Figure 1C, LPS significantly increased the expression of miR-25, miR-125a, miR-125b, miR-146a, miR-146b, miR-155 and miR-455.

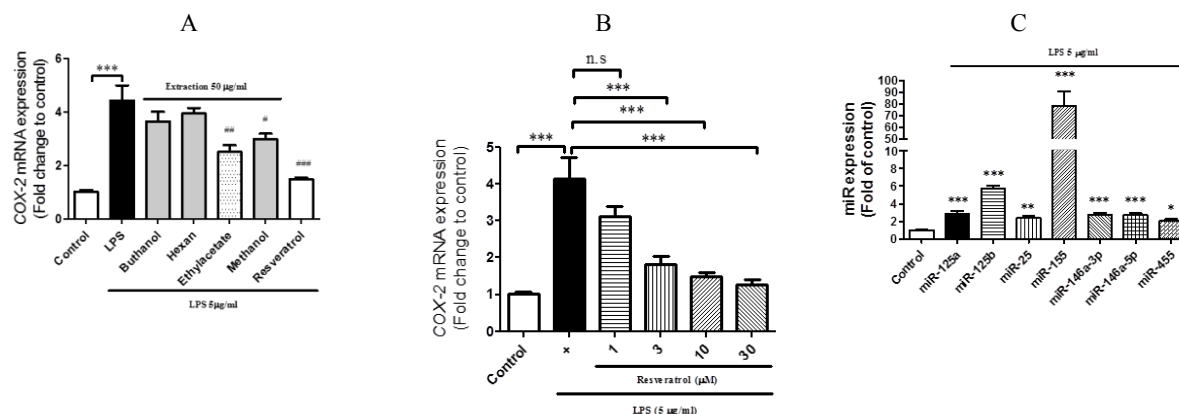


Figure 1.

Comparison of COX-2 expression effects between four fractions 50 $\mu\text{g}/\text{mL}$ and 30 μM resveratrol isolated from *Vitis heyneana*. Eighteen hours after treating cells with LPS (5 $\mu\text{g}/\text{mL}$) with or without the fraction, on Raw264.7 cells. (A) Samples were collected for qPCR. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *significant as compared to control; #significant as compared to LPS group; $n = 6$). (B) Dose-dependent effect of resveratrol on COX-2 mRNA expression (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 6$). (C) Effect of LPS (5 $\mu\text{g}/\text{mL}$) on miRs expression (miR-125a, miR-125b, miR-25, miR-155, miR-146a-3p, miR-146a-5p and miR-455) on Raw264.7 cells by qPCR (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 6$).

The stilbenes and oligostilbenes isolated from natural plants have diverse pharmacological effects such as anti-cancer [13], anti-oxidant [14], anti-aging [15], anti-inflammatory or anti-atherosclerotic [16]. Among them, resveratrol has been considered as one of the most important active components. Recently, Wang *et al.* depicted that resveratrol inhibits glioma cell growth through miRNAs expression including: miR-21, miR-30a-5p and miR-19 [17] and reduced COX-2 expression.

miRNAs are non-coding RNAs (18-25 nucleotides length) that bind to the 3'UTR of certain mRNAs and regulate mRNA stability and protein synthesis. miRNAs mediate either translational repression/

activation or degradation of the target mRNA [18]. During the last decade, miRNAs have been suggested as a new mechanism involved in a number of human pathological states that are showed in over 45,000 papers (from PubMed database).

To further examine the effect of resveratrol on these miRNAs, cells were treated with resveratrol (10 μM) for 24 hr and subjected to qPCR. Figure 2A depicts that only miR-146a-5p was inhibited by resveratrol, this compound having no effect on other miRNAs.

To address the role of miR-146a-5p on LPS-induced COX-2 expression response to resveratrol, miR-146a-5p mimic was transfected and then stimulated with LPS and resveratrol for 24 hr. As expected,

miR-146a mimic significantly induced COX-2 protein and mRNA expression. In addition, resveratrol both potently abrogated LPS- and miR-146a mimic-induced COX-2 expression in Raw264.7 cells

(Figure 2B and Figure 2C). These data suggested the role of miR-146a-5p in resveratrol suppressed LPS-induced COX-2 in macrophages.

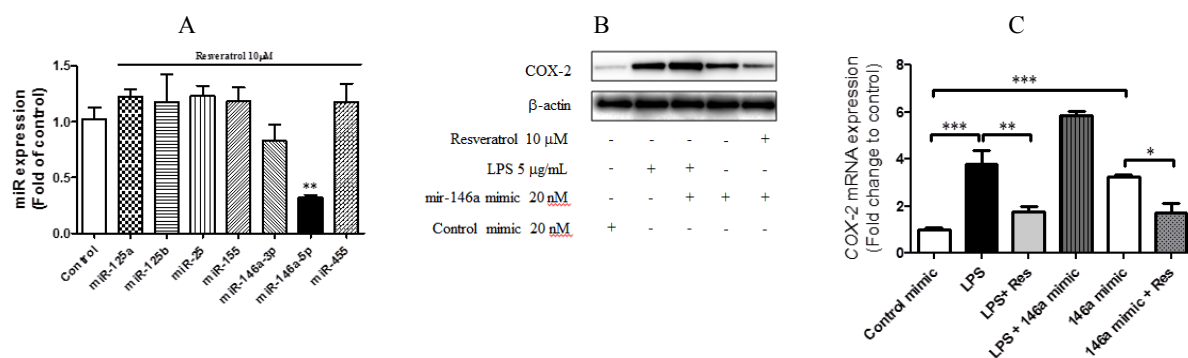


Figure 2.

Role of miR-146a-5p in resveratrol inhibited LPS-induced COX-2 expression. (A) Effect of resveratrol on miRNAs expression (miR-125a, miR-125b, miR-25, miR-155, miR-146a-3p, miR-146a-5p and miR-455) on Raw264.7 cells by qPCR (** $p < 0.01$; $n = 6$). (B, C) Effect of miR-146a-5p mimic and resveratrol on LPS-induced COX-2 expression. Raw264.7 cells were transfected with 20 nM of negative control, miR-146a-5p mimic for 96 h and then stimulated with or without LPS and resveratrol for 18 h. Cells were then harvested for qPCR and Western blot analysis (** $p < 0.001$; $n = 6$).

Conclusions

In our current report, we isolated resveratrol from *Vitis heyneana* and demonstrated it inhibited miR-146a-5p expression. Moreover, LPS-induced COX-2 expression was suppressed by resveratrol involving miR-146a-5p inhibition. These data first demonstrated the role of miR-146a in resveratrol response to COX-2 expression.

Conflict of interest

The authors have declared that there is no conflict of interest.

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