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Oridonin inhibits vascular inflammation by blocking NF-κB and MAPK activation

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ABSTRACT

Oridonin, an active diterpenoid compound isolated from the plant Rabdosia Rrubescens, has various pharmacological activities, including antioxidant, anti-tumor capacities and anti-inflammation. In the present study, we explore the role of oridonin in regulating endothelial inflammation and its underlying mechanism. Endothelial cell-monocyte interaction was detected by Leukocyte-endothelium Adhesion Assay. The protein expression was measured by Western blot. NF-κB p65 translocation was measured by immunofluorescence. Acute lung inflammation model was used to evaluate leukocyte infiltration in vivo. The endothelial-leukocyte adhesion and the leukocyte transmigration were profoundly reduced by oridonin. Oridonin dramatically inhibited the expression of TNF-α-induced endothelial adhesion molecules (intercellular adhesion molecule-1 (ICAM-1); vascular cell adhesion molecule-1 (VCAM-1) and E-selectin) and the pro-inflammatory cytokine (IL-6, IL-8 and monocyte chemoattractant protein-1(MCP-1)). Oridonin suppressed the penetration of the leukocyte in the acute lung injury mice model. Furthermore, Oridonin also suppressed the TNF-α-activated MAPK and Nuclear factor kappa B (NF-κB) activation. Our results suggest that oridonin has the anti-inflammatory properties in endothelial cells, at least in part, through the suppression of MAPK and NF-κB activation, which may have a potential therapeutic use for inflammatory vascular diseases.

1. Introduction

The endothelium forms a barrier between the vessel lumen and the surrounding tissue. Vascular endothelial cells play an important role in the regulation of vascular permeability, vascular tone, vascular inflammatory response and angiogenesis (Cines et al., 1998; Pober and Sessa, 2007). Endothelial dysfunction is associated with the development of inflammatory disorders states, including tumor, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease and sepsis (Aird, 2007; Gareus et al., 2008; Khan et al., 2010; Roifman et al., 2009). Activation of the endothelium at sites of inflammation aggravates endothelial dysfunction and produces adhesion molecules and multiple cytokines, which could promote angiogenesis (Tian et al., 2009). A series of adhesion molecules, particularly vascular cell adhesion molecule1(VCAM-1), intercellular adhesion molecule 1 (ICAM-1), E-selectin and cytokines such as MCP-1, IL-6 and IL-8 (Robinson, 2014) are expressed in inflammatory disorders, by which the blockade reduces disease in models of multiple sclerosis, inflammatory bowel disease, and asthma (Cook-Mills et al., 2011). Therefore, medications that suppressing the expression of pro-inflammatory cytokines and adhesion molecules are promising candidates for the treatment and prevention of chronic inflammatory diseases.

Oridonin, an active diterpenoid compound isolated from the plant Rabdosia Rrubescens, was found to possesses various physiological and pharmacological outcomes, such as anti-tumor, anti-bacteria, anti-inflammation, scavenging active oxygen free radicals and anti-mutagenetic effects (Han et al., 2004). Many studies have showed that oridonin was able to induce autophagy of a number of human cancer cell types, and have demonstrated remarkable anti-poliferative and pro-apoptotic effects against leukemia and some solid tumors (Guo et al., 2012), including hepatocellular carcinoma, gastric cancer, colorectal cancer, breast cancer, anrd pancreatic cancer (Bao et al., 2014; Kuo et al., 2014). Recent studies have demonstrated that oridonin was beneficial influences on immune balance and inhibits the release of

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proinflammatory mediators (Al-Amran et al., 2014; Ku and Lin, 2013). However, whether oridonin could have a potential role to affect vascular inflammation has not been clarified. In the current study, we investigated whether oridonin could inhibit TNF-α–induced vascular inflammation in human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Cell culture

The human umbilical vein endothelial cells (HUVECs) had been isolated from umbilical veins which were obtained from the first affiliated hospital of sun yat-sen university, and cultivated as described previously. Cell isolation was approved by the first affiliated hospital of Sun Yat-sen University ethics committee. HUVECs were cultured in EBM2 media supplemented with various growth factors and 2% FBS (Lonza,CA, USA) when the cells reached 80–90% confluence. To avoid cell aging, all experiments were carried out by using HUVEC between passages 3 and 5. The cells medium was replaced every 2 days, and the cells were grown at 37 °C under 5% CO_2.

2.2. Cell viability assay

HUVECs were seeded in a 96-well plate and were incubated with either 0.1% dimethylsulfoxide (DMSO) as a diluent control or the indicated concentration oridomin for 48 h. The number of viable cells was evaluated by the MTT method. Briefly, after the incubation period, the culture mediums from each well were removed and the adherent cells were incubated with 20 μl of 5 mg/ml MTT (Sigma-Aldrich,USA) solution for 4 h. After the used media containing MTT solution was removed, MTT formazan was dissolved in dimethyl sulfoxide (DMSO). The plates were read at 540 nm in a microplate reader (BioRad, Hercules, CA). Cell viability in the control group was set at 1, and cell viability in the other groups was determined as a fold of the control.

2.3. Monocyte-HUVEC adhesion assay

Monocyte-HUVEC adhesion assay was performed by Cell Biolabs' CytoSelect™ Leukocyte-endothelium Adhesion Assay. Human umbilical vein endothelial cells (HUVECs) were grown in Gelatin-coated 24-well plates at 80–90% confluence. HUVECs were pretreated with oridonin for 24 h, and stimulated with 10 ng/ml TNF-α for 4 h. Then, LeukoTracker™ solution–loaded leukocytes were added to each well. The PBMC were incubated with the LeukoTracker™ solution for 1 h at 37 °C, and washed twice with growth medium. This was followed by adding of the labeled PBMC to the HUVECs. The non-adherent cells were removed from the plate by washing with PBS after incubation. Count the adherent leukocytes under an inverted fluorescence microscope; at least three separate fields per well on average. Aspirate the final wash and add Lysis Buffer to each well containing cells and fluorescence was read by using a fluorescence plate reader at 480 nm/520 nm. The adhesion data was represented in terms of the percentage change compared with the TNF-α value.

2.4. RNA isolation and quantitative polymerase chain reaction

The confluent monolayers of the HUVECs were treated with oridonin for 24 h and then stimulated with TNF-α for 12 h; total RNAs were isolated from HUVECs by using TRIzol (Sigma) and were prepared by Takara PrimeScript® RT reagent kit according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) analysis for the expression of VCAM-1, ICAM-1, E-selectin, IL-6, IL-8, and MCP-1 were performed on cDNA with QuantiTect SYBR Green RT-PCR Kit by using the StepOnePlusTM Real Time PCR System (Applied Biosystems). Relative gene expression was normalized to the expression of GAPDH as endogenous control. The RT-PCR primers used are listed in Supplementary Table 1 (STable 1).

2.5. Detection of cell culture supernatant level of IL-6 , IL-8 and MCP-1

To ascertain whether oridonin affected IL-6 , IL-8 and MCP-1 release from HUVECs, cells were collected after being treated with or without oridonin and then treatment with TNF-α for 12 h. Cell culture supernatants were collected for the determination of IL-6 , IL-8 and MCP-1 by using ELISA kit (R&D Systems, USA).

2.6. Western blot analysis

Cells were harvested and lysed in ice-cold radioimmunoprecipitation (RIPA) buffer containing protease inhibitors and phosphatase inhibitors (Thermo Scientifical), and total protein concentrations in the supernatant were determined using the BCA Protein Assay Kit following manufacturer's instructions. Western blot analysis was performed by using a standard protocol. The equal amounts of samples were separated on 12% SDS-PAGE gel and electrophoretically transferred onto Nitrocellulose membranes (Bio-Rad, USA). The membranes were immunoblotted with the indicated antibodies: anti-VCAM1(Abcam), anti-ICAM1(Abcam), anti-E-selectin(Abcam) anti-JNK (Cell signaling), anti-phospho-JNK(Cell signaling), anti-p38(Cell signaling), anti-phospho-p38(Cell signaling), anti-ERK(Cell signaling), anti-phosphor-ERK(Cell signaling), anti-NF-κB p65(Cell signaling), anti-phosphor-IKBα(Cell signaling), anti-IKBα(Cell signaling) and anti-phosphor-IKK(Cell signaling). Each blot is a representative of at least three similar independent experiments. Densitometry analysis was conducted by using the Image-Pro Plus software version 6.0 (Media Cybernetics Corp, Bethesda, MD, USA) and normalized by the housekeeping protein b-actin.

2.7. Model of acute lung inflammation

Male C57BL/6 J mice were randomly pretreated with DMSO or oridonin (35 mg/kg weight, once a day) by intraperitoneally for 7 days. Recombinant mouse TNF-α (2 μg/mouse) was injected intraperitoneally to induce lung injury. Five hour after TNF-α, all animals were killed to harvest tissues and organs for analysis. Lungs were used for H&E staining. An Amplex Red Peroxidase Assay Kit (Invitrogen) was used to detect myeloperoxidase (MPO) activity in homogenates of murine lung tissue.

2.8. Confocal laser scanning fluorescence microscopy

Cells were plated into glass coverslips and grown with EBM-2 and reagents for 24 h, respectively. Then, cells were treated with oridonin or DMSO for 24 h and then stimulated with TNF-α for 30 min and they were fixed in absolute methanol and washed with PBS containing 0.4% Tween-20. After washing, cells were blocked with PBS containing 10% goat serum, followed by incubation with rabbit polyclonal anti-p65 antibodies overnight. Cells were then incubated with second antibody and then incubated with DAPI and the coverslips were mounted on glass slides with antifade mounting media and examined using a confocal fluorescence microscope (Zeiss LSM710).

2.9. Study approval

The human study protocol was approved by the Medical Ethical Committee of the First Affiliated Hospital at Sun Yat-sen University and the Guiyang College of Traditional Chinese Medicine and was conducted according to the *recommendations of the Declaration of Helsinki and the Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, and Vol. 2, 1984, Canadian Council on Animal Care (CCAC),*www.ccac.ca*)*. All patients provided informed consent to participate in the study. The experimental protocols for animal care and use were