Vascular Pharmacology 72 (2015) 43–52

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Wogonin inhibits LPS-induced vascular permeability via suppressing MLCK/MLC pathway

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ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 9 April 2015
Accepted 20 April 2015
Available online 5 May 2015

Chemical compounds studied in this article:
Wogonin (PubChem CID: 5281703)
Wortmannin (PubChem CID: 312145)

Keywords:
Wogonin
Lipopolysaccharide (LPS)
Vascular permeability
MLCK/MLC

ABSTRACT

Wogonin, a naturally occurring monoflavonoid extracted from the root of *Scutellaria baicalensis* Georgi, has been shown to have anti-inflammatory and anti-tumor activities and inhibits oxidant stress-induced vascular permeability. However, the influence of wogonin on vascular hyperpermeability induced by overabounded inflammatory factors often appears in inflammatory diseases and tumor is not well known. In this study, we evaluate the effects of wogonin on LPS induced vascular permeability in human umbilical vein endothelial cells (HUVECs) and investigate the underlying mechanisms. We find that wogonin suppresses the LPS-stimulated hyperactivity and cytoskeleton remodeling of HUVECs, promotes the expression of junctional proteins including VE-Cadherin, Claudin-5 and ZO-1, as well as inhibits the invasion of MDA-MB-231 across EC monolayer. Miles vascular permeability assay proves that wogonin can restrain the extravasated Evans in vivo. The mechanism studies reveal that the expressions of TLR4, p-PLC, p-MLCK and p-MLC are decreased by wogonin without changing the total steady state protein levels of PLC, MLCK and MLC. Moreover, wogonin can also inhibit KCl-activated MLCK/MLC pathway, and further affect vascular permeability. Significantly, compared with wortmannin, the inhibitor of MLCK/MLC pathway, wogonin exhibits similar inhibition effects on the expression of p-MLCK, p-MLC and LPS-induced vascular hyperpermeability. Taken together, wogonin can inhibit LPS-induced vascular permeability by suppressing the MLCK/MLC pathway, suggesting a therapeutic potential for the diseases associated with the development of both inflammatory and tumor.

1. Introduction

Vascular hyperpermeability is a common pathogenic process which is often found during the development of several malignant diseases such as diabetic retinopathy [53], solid tumors [35], myocardial infarction [54], wounds [15], and chronic inflammation [28]. The determining structure of vascular permeability [34] is endothelial integrity, which is tightly controlled by many types of signals such as physiological factors (VEGF, TNF or INFγ), oxidative stress and pro-inflammatory cytokines [1,20]. Lipopolysaccharide (LPS), an important cell wall component of Gram-negative bacteria, is thought to be the major pathogenic factor involved in inflammation and sepsis and produced by inflammatory mediators and inflammatory cytokines from immunocytes [12]. In addition, LPS plays an important role in regulating vascular permeability [59]. It has been shown that with the stimulation of LPS, stress fiber of ECs contracted and intercellular space turned larger, then macromolecules and cells could get across the EC monolayer [27,46]. In severe trauma and sepsis, vascular hyperpermeability could result in protein-rich tissue edema, abnormalities of the internal environment, abdominal compartment syndrome and multiple organ dysfunction syndrome [8], as well as the morbidity and mortality in bacterial sepsis [40]. Therefore, stabilizing junction between ECs, decreasing vascular permeability and normalizing function of blood vessel are critical for the therapy of these diseases and new strategies for drug development.

LPS binds to toll-like receptor 4 (TLR4) in ECs, which in turn activates the downstream signaling pathways and generates the contraction of cytoskeleton, migration of ECs and finally increasing endothelial permeability [49]. Myosin light chain kinase (MYLK or MLCK) is a crucial regulator of endothelial contractility [19,39], whose nonmuscle isoform is a Ca^{2+}-calmodulin-dependent enzyme presented in ECs and plays a key role in inducing endothelial permeability [18,41,50]. Myosin light chain (MLC), the phosphorylation of which depends on the activation

Abbreviations: LPS, lipopolysaccharide; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1; MLCK (MYLK), myosin light chain kinase; MLC, myosin light chain; p-MLCK, phosphorylated myosin light chain kinase; p-MLC, phosphorylated myosin light chain; TLR4, toll-like receptor 4; PLC, phospholipase C; p-PLC, phosphorylated phospholipase C; HUVECs, human umbilical vein endothelial cells.

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http://dx.doi.org/10.1016/j.vph.2015.04.012
1537-1891/

of MLCK [7,47], can reorganize the cytoskeleton and tether forces in the intercellular junctions [48]. The involvement of MLCK in MLC phosphorylation is dependent on the activation of Phospholipase C (PLC) [37]. Phosphorylated PLC triggers phosphoinositide hydrolysis, resulting in inositol 1,4,5-trisphosphate (IP3)-mediated calcium mobilization, then the increased intracellular calcium concentration leads to MLCK activation, thus increasing MLC phosphorylation [42]. Activation of TLR4 signaling transduction in ECs can activate PLC, increase intercellular Ca^{2+} concentration, stimulate MLCK/MLC pathway and induce vascular hyperpermeability [58].

The Chinese herb Huang Qin, *Scutellaria baicalensis* Georgi which has a high flavonoid content [22], has been traditionally widely used in treating several diseases such as inflammation, diarrhea, and pyogenic infections [32]. Wogonin is one of the major flavonoids which are the most common active ingredients in the root of *S. baicalensis* Georgi [45]. It has been widely studied and proved to possess a wide spectrum of pharmacological properties, such as antioxidant [17], anti-tumor [25], and anti-inflammatory [55] activities both in vitro and in vivo. In the previous study, we have proved that wogonin could inhibit LPS induced tumor angiogenesis via Wnt/β-catenin pathway [60] and decrease H_2O_2-induced vascular permeability [51]. However, there was certain limitation in using H_2O_2, an exogenous stimulating factor, to simulate the microenvironment in vivo and the molecular mechanisms of the effect of wogonin on decreasing vascular hyperpermeability were not explored in depth. In addition, whether wogonin could affect vascular hyperpermeability induced by some common cytokines, which are overabounded in inflammatory and tumor microenvironment generally, such as LPS, along with the molecular mechanisms, remains unknown and further investigations are needed.

In our studies, we have evaluated the effects of wogonin on LPS-induced vascular permeability and explored the underlying mechanisms. LPS activates HUVECs leading to hypermotility and vascular hyperpermeability. Herein, we elucidate the potential effects of wogonin on the response of HUVEC migration, cytoskeleton remodeling, junctional protein expression and MDA-MB-231 transendothelial cell migration in vitro, in addition, the extravasated Evans in vivo. As a result, we find that wogonin inhibits LPS-induced vascular permeability obviously in vitro and in vivo. Further mechanism researches reveal that wogonin inhibits vascular permeability via influencing the MLCK/MLC pathway, and regulates the expression levels of TLR4, p-PLC, p-MLCK and p-MLC in LPS-stimulated HUVECs. Taken together, all the results suggest that wogonin can decrease the vascular hyperpermeability and might serve as a candidate in the development of human diseases associated with vascular hyperpermeability therapy such as inflammatory and tumor.

2. Materials and methods

2.1. Materials

Wogonin was isolated from *S. baicalensis* Georgi according to the protocols reported previously, with slight modifications [24]. Samples containing 99% or higher wogonin were used in all experiments unless otherwise indicated. For our experiments, wogonin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Primary antibodies for ZO-1, TLR4, p-PLC, p-MLCK, p-MLC and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for VE-Cadherin, p-VE-Cadherin, Claudin-5, PLC, MLC and MLCK were obtained from Bioworld (St. Louis Park, MN). IRDye™ 800 conjugated anti-mouse/rabbit second antibody was purchased from Rockland Inc. (Philadelphia, PA). Bovine serum albumin (BSA), paraformaldehyde, Triton X-100, Tris, NaCl, EDTA, NP-40, PMSF, NaF, SDS, DTT, LPS, KCl, DAPI, Evans blue and fluorescein-5-isothiocyanate (FITC)-conjugated phalloidin were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

The human breast carcinoma cell line MDA-MB-231 was originally obtained from the Cell Bank of Shanghai Institute of Cell Biology. MDA-MB-231 cells were cultured in L-15 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 U/ml streptomycin, pH 7.4. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously [26]. The harvested cells were grown in medium 199 (Gibco, Grand Island, NY) containing endothelial cell growth supplement (ECGS, 30 μg/ml; Sigma, St. Louis, MO), epidermal growth factor (EGF, 10 ng/ml; Sigma, St. Louis, MO), 20% fetal bovine serum (FBS, Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 U/ml streptomycin, pH 7.4. After 3–5 passages, HUVECs were collected for use in all experiments. All cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Endothelial cell migration assay

Chemotactic motility of HUVECs was assayed using a Transwell chamber (Millicell, Billerico, MA) as described previously [52]. HUVECs were treated with wogonin (0, 1, 10 and 100 μM) as indicated for 4 h. Then cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in serum-free M199. Cell suspension was loaded into each of the upper wells and M199 containing 1% fetal bovine serum with or without LPS (1 μg/ml) was added into the lower compartment. Following incubation at 37 °C in 5% CO_2 for 4 h, nonmigratory cells on the upper surface were removed by a cotton swab. The migrated cells on the lower surface were fixed with 100% methanol and stained with hematoxylin and eosin. The migrated cells were quantified by manual counting and five randomly chosen fields were analyzed for each group.

2.4. Immunofluorescence staining of the actin cytoskeleton

HUVECs grown on coverslips were pretreated with wogonin (0, 1, 10, and 100 μM) for 4 h and stimulated with or without LPS (1 μg/ml) for 1 h as indicated after being washed with PBS twice. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized for 10 min in 0.2% Triton X-100. Then they were incubated with 3% BSA in PBS to block nonspecific binding. Cells were subsequently processed by incubation with FITC-conjugated phalloidin (specific for F-actin staining), 1:30 dilution for 1 h at 37 °C. Then, cells were washed three times with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI). Finally, the slips were mounted with anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and photographed with a confocal laser scanning microscope (Fluoview FV 1000, Olympus, Tokyo, Japan).

2.5. Transendothelial cell migration assay

The experiment was performed as previously described [16]. Briefly, HUVECs were seeded in the amount of 1×10^4 cells on Transwell polycarbonate filters (6.5 mm in diameter, 8 mm pore-size, Corning-Costar, Cambridge, MA). After reaching a confluence, cells were preincubated for 4 h with wogonin (0, 1, 10 and 100 μM) and then stimulated with or without LPS (1 μg/ml) for 1 h as indicated after being washed with PBS twice. Then MDA-MB-231 cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in L-15 serum-free medium. Cell suspensions were placed in the upper compartment, and medium with 10% fetal bovine serum was added in the lower compartment. Following incubation at 37 °C in 5% CO_2 for 4 h, nonmigratory cells on the upper surface were removed with a cotton swab. The migrated tumor cells on the lower surface were fixed with 100% methanol and stained with hematoxylin and eosin. The migrated cells were quantified by manual counting and five randomly chosen fields were analyzed for each group.