International Immunopharmacology 63 (2018) 261–269

Contents lists available at ScienceDirect

International Immunopharmacology

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

Attenuation of antimalarial agent hydroxychloroquine on TNF-α-induced endothelial inflammation

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ARTICLE INFO

Keywords:
Hydroxychloroquine
Endothelial cells
Inflammation
NF-κB
MAPK

ABSTRACT

Objective: Hydroxychloroquine (HCQ) is an antimalarial drug that is widely used in the treatment of some autoimmune diseases. In the present study, we explore the role of HCQ in regulating endothelial inflammation and its underlying mechanism.

Methods: Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords. Protein expression was measured by Western blot or immunofluorescence staining. Endothelial adhesion ability was determined by leukocyte-endothelial monolayer adhesion assay. Transwell assay was used to measure the transendothelial-migration of PBMCs.

Results: TNF-α-induced endothelial-leukocyte adhesion and the leukocyte transmigration were profoundly reduced by HCQ treatment. HCQ treatment dramatically inhibited the expression of TNF-α-induced endothelial ICAM-1 and VCAM-1. Furthermore, treatment with HCQ prevented the TNF-α-induced translocation of NF-κB p65 into the nucleus and the phosphorylation of the p65 subunit in HUVECs. HCQ inhibited the expression of phosphorylated p38 and JNK protein but not ERK. Treatment with NF-κB, p38 and JNK inhibitor could also reduce TNF-α-induced endothelial-leukocyte adhesion and the endothelial expression of ICAM-1 and VCAM-1. HCQ administration also suppressed TNF-α induced lung injury in mice by reducing neutrophil infiltration in pulmonary interstitial tissue.

Conclusions: This work shows the inhibitory effect of HCQ on endothelial inflammatory response through, at least in part, blocking NF-κB, p38 and JNK pathways. Our findings suggest that HCQ may be a promising approach for the treatment of inflammatory vascular disease beyond its immunomodulatory actions.

1. Introduction

Accumulating evidence indicates that chronic inflammation contributes to the initiation and propagation of endothelial dysfunction. Endothelial inflammation plays a critical role in the accelerated atherosclerosis and higher cardiovascular morbidity and mortality, particularly in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), spondyloarthritis, diabetes mellitus and others chronic inflammatory diseases [1–3].

In normal conditions, the endothelium lines the lumen of blood vessels and acts as a physical barrier that performs physiologic functions by regulating cellular adhesion and resistance to thrombosis. However, in pathologic circumstances, some pro-inflammatory cytokines, particularly TNF-α, may trigger endothelial dysfunction by upregulating endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), and several chemokines, and leading to the increased adhesion of leukocytes to the endothelium and the trans-endothelial migration [4–6].

TNF-α-induced endothelial inflammation is controlled by several

Abbreviations: HCQ, hydroxychloroquine; EC, endothelial cell; HUVEC, human umbilical-vein EC; TNF-α, tumor necrosis factor-α; PBMC, peripheral blood mononuclear cell; JNK, c-Jun N-terminal kinase; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1

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https://doi.org/10.1016/j.intimp.2018.08.008
Received 28 April 2018; Received in revised form 1 August 2018; Accepted 6 August 2018

signaling pathways. Endothelial activation of the NF-κB pathway is critical for modulating expression of a series of inflammation-related genes that encode adhesion molecules and proinflammatory cytokines [7–9]. Targeting the NF-κB pathway has been considered as a novel strategy to control endothelial inflammation. MAPK pathways consist of a family of highly conserved serine/threonine protein kinases, which include three major classes in mammals, the extracellular signal-regulated kinases (ERKs), p38 and c-Jun N-terminal kinase (JNK) [10]. Previous studies indicate that MAPKs are also important for TNFα-induced endothelial inflammation [11–14].

Hydroxychloroquine (HCQ), an antimalarial compound with low cost and rare side effects, is an effective immunomodulatory agent that has been used in the treatment of SLE and RA for many years. HCQ has also been reported to improve thrombo-vascular events [15], experimental antiphospholipid syndrome (APS) [16,17] and arteriosclerosis [18,19]. The drug could prevent the development of experimental pulmonary hypertension [20] and decrease the vascular oxidative stress in mice with lupus [21]. HCQ has also been shown to exert protective endothelial effects as adjuvant therapy for preeclampsia [22]. These studies suggest that HCQ might have potential roles for improving atherosclerosis and cardiovascular disease in rheumatic diseases beyond its immunomodulatory actions. However, to date, the underlying mechanisms for the protective effects of HCQ on endothelial cell-mediated inflammatory events in rheumatic diseases are still unclear. Therefore, in the present study, we determined the role of HCQ in regulating TNF-α-induced endothelial inflammation and explored its underlying mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). Cell culture reagents, including DMEM/Ham's F12 (DMEM/F12), fetal bovine serum, antibiotics, trypsin-EDTA and PBS, were purchased from Invitrogen (Carlsbad, CA, USA). EBM2 and EGM2 media were obtained from LONZA (USA). Hydroxychloroquine, collagenase, NF-κB inhibitor Triptolide, p38 inhibitor SB203580, JNK inhibitor SP600125, and anti-β-actin antibody were purchased from Sigma Chemicals (St Louis, MO, USA). Anti-JNK and anti-phospho-JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies for anti-ICAM-1, anti-VCAM-1, anti-p38, anti-phospho-p38, anti-ERK, and anti-phospho-ERK were purchased from Abcam (Cambridge, MA, USA).

2.2. Cell culture

For preparing human umbilical vein endothelial cells (HUVECs), fresh human umbilical cords were obtained from 8 single postpartum women who have no infection with HIV-1, hepatitis B and hepatitis C, and treated with collagenase. HUVECs were isolated and incubated in EGM2 media containing various growth factors and 2% FBS. The cells were grown in a humidified incubator at 37 °C with 5% CO2. HUVECs were passaged when the cells reached 80–90% confluency. Flow cytometric analysis indicated that 99.91% cells express the HUVECs surface marker CD31 (Suppl Fig. S1). HUVECs were collected for further experiments from passage 4 to 6.

2.3. Leukocyte-endothelial monolayer adhesion assay

This assay was performed using the CytoSelect™ Kit (Cell Biolabs). HCQ (10 μmol/L) was added to the designated wells and incubated for 24 h to 80–90% confluence. Then, 10 ng/mL of TNF-α was added into the designated wells and incubated for another 6 h. LeukoTracker™ solution–loaded human peripheral blood leukocytes were added to each well and incubated for 1.5 to 2 h. Nonadherent leukocytes were removed, and the adherent cells were counted under an inverted fluorescence microscope. The average cell counts of at least three separate fields per well were quantified.

2.4. Transwell assay for the measurement of trans-migration of PBMCs

Transwell migration assay was conducted by the Boyden chamber method in 24-well plates with inserts of 6.5 mm diameter and 8-micron pore size (Transwell, Corning Inc., USA). The top chambers were filled with 200 μL EBM-2 medium containing various growth factors and 2% FBS. HUVECs were seeded to the top chamber and incubated to 100% confluency, and then were grown in EBM-2 medium without growth factors and FBS for 12 h. HUVECs were pretreated with various concentrations of HCQ for 24 h, and then, TNF-α (10 ng/mL) was added, followed by 4 h of incubation. Human PBMCs were seeded on to the top chambers and incubated for 2 h. The medium in the bottom chambers was collected, and the number of migrated PBMCs was counted by flow cytometry. The assays were replicated 3 times.

2.5. Western blot analysis

Cells were lysed with lysis buffer (CST) for 15 min on ice, and lysates were centrifuged for 15 min at 12,000 r.p.m. and 4 °C to harvest the supernatants. The protein concentrations were measured by a BCA protein assay (Pierce, Rockford, IL, USA). Supernatants were incubated with 2 × Laemmli sample buffer (Sigma) at 100 °C for 5 min. The equal amounts of samples were then separated with SDS-PAGE gel, transferred on NC membranes and immunoblotted with the indicated antibodies: anti-VCAM-1, anti-ICAM-1, anti-JNK, anti-phospho-JNK, anti-phosphor-p38, anti-p38, anti-phosphor-ERK and anti-ERK.

2.6. Fluorescence microscopy

Human umbilical vein endothelial cells (HUVECs) on glass coverslips at 80–90% confluency were treated with HCQ or DMSO for 8 h, and then, they were stimulated with TNF-α for 30 min. Cells were fixed with paraformaldehyde and permeated with 0.1% TritonX-100 in PBS. For determination of NF-κB p65, the cells were incubated with anti-p65 antibody or overnight and incubated with secondary antibodies for 1 h at room temperature. The cells were then incubated with DAPI, and the coverslips were mounted on glass slides with antifade mounting media and examined using fluorescence microscopy (Zeiss LSM710).

2.7. Administration of HCQ in TNF-α-induced lung injury mouse model

Neutrophil recruitment into the lung was analyzed in a murine model of TNF-α-induced pulmonary inflammation [23,24]. The nude mice (6–8 weeks, $n = 24$) were randomly divided into three groups including the sham, TNF-α alone and HCQ ($3\ mg{\cdot}kg^{-1}$ day) + TNF-α. Mice were pretreated with DMSO or HCQ twice a day for a week by intraperitoneal injection. The TNF-α group (TNF-α) and HCQ + TNF-α group mice were intraperitoneally (i.p.) injected at a dosage of $2\ mg{\cdot}kg^{-1}$ TNF-α [23] 4 h before sacrifice, whereas the sham group was treated with normal saline (NS, $1.5\ mL{\cdot}kg^{-1}$). The lungs of mice with different treatments were fixed in 4% neutral-buffered formalin and embedded in paraffin. Sections (3 μm thick) were stained with hematoxylin and eosin (H&E).

2.8. Study approval

The human study protocol was approved by the Medical Ethical Committee of the First Affiliated Hospital at Sun Yat-sen University and was conducted according to the *recommendations of the Declaration of Helsinki*. All patients provided informed consent to participate in the study.