Original Paper

Crocetin Inhibits Lipopolysaccharide-Induced Inflammatory Response in Human Umbilical Vein Endothelial Cells

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Key Words

Crocetin • HUVEC • Vascular inflammation • MCP-1 • IL-8 • NF-κB p65

Abstract

Background/Aim: Crocetin is a readily bioavailable and bioactive compound extracted from *Saffron*. Previous studies indicated its various biomedical properties including antioxidant and anti-coagulation potencies. However, its effect on inflammation, notably within the cardiovascular system, has not been investigated yet. In the present study, we utilized human umbilical vein endothelial cell (HUVEC) to elucidate the effect of Crocetin on vascular inflammation. **Methods:** Cell viability and toxicity were evaluated by MTT and Lactate dehydrogenase (LDH) assay, respectively. Pro-inflammatory chemokine Monocyte Chemoattractant Protein-1 (MCP-1) and Interleukin-8 (IL-8) expressions were determined by RT-PCR and ELISA. With fluorescence labeled U937 cells, we examined immune cell adhesion to the inflamed HUVEC *in vitro*, which was further confirmed by the H&E staining in the murine subcutaneous endothelium *in vivo*. **Results:** Upon Lipopolysaccharide (LPS)-induced inflammatory response in HUVECs, Crocetin ameliorated cell cytotoxicity, suppressed MCP-1 and IL-8 expressions through blocking NF-κB p65 signaling transduction. Moreover, Crocetin inhibited immune cells adhesion and infiltration to inflamed endothelium, which is a key step in inflammatory vascular injury. **Conclusions:** These findings suggest that Crocetin, a natural herb extract, is a potent suppressor of vascular endothelial inflammation.

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Introduction

Crocetin is an ingredient originally discovered in dried stigma of *Crocus Sativus* (*Saffron*) [1], a herb served as traditional Chinese medicine over centuries. Recently, it draws attention in biomedical research due to Crocetin's ready bioavailability with few side effects. So far,

accumulative evidence reveals its role on anti-cancer, anti-asthma, anti-oxidative stress, and anti-atherosclerosis [2-5].

Inflammation plays a critical role in cardiovascular disease (CVD) such as atherosclerosis [6-8]. Typically, during the initiation stage of atherogenesis, inflammation occurs in vascular endothelium, the innermost layer of the vessel. Inflamed endothelial cells release pro-inflammatory mediators, such as pro-inflammatory cytokines, chemokines, and adhesion molecules [9, 10]. Recruited by these mediators, circulatory monocyte adheres to and infiltrate the lesion site, triggers subsequent pathogenesis process including excessive lipid accumulation, tunica smooth muscle cell migration, and plaque formation, results in amplification of vascular inflammation[6]. Two well-studied chemokines, MCP-1 and IL-8, are previously reported regulating immune cell adhesion, and integration with endothelial cell process [11-13], thus become potential therapeutic targets which attract research attention. Furthermore, production of those pro-inflammatory chemokines is mediated by transcription factor NF- κ B, an inflammatory-related signaling cascade which is activated during endothelium inflammation [14, 15]. So far, to our knowledge, Crocetin's anti-inflammatory effect and its mechanism, have not been reported yet.

In present study, we aim to examine the therapeutic effect of Crocetin in vascular inflammation via its role in inhibition MCP-1 and IL-8. Of note, due to its high cost of *Saffron*, the fruit of *Gardenia Jasminoides Ellis*, a member of *Rubiaceae* family, is used as an alternative source for Crocetin extraction[16].

Materials and Methods

Preparation of Crocetin

Crocetin (>98%, high-performance liquid chromatography, HPLC) was prepared by our group as previously described (Fig. 1)[16].

Cell culture

Human umbilical vein endothelial cell (HUVEC) line was generously offered by Dr. Vincent Moy (Miller School of Medicine, University of Miami); U937 human leukemic monocyte lymphoma cell line was purchased from Institution of Biochemistry and Cell Biology, SIBS (Shanghai, China). HUVECs were maintained in RPMI medium 1640 (GIBCO), and supplemented by Endothelial Cell Growth Kit (Lonza); U937 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum and 100 Unit/mL Penicillin and Streptomycin. A Trypan Blue (GIBCO) Staining was performed to guarantee >95% live cells before experiments. Both cell lines were maintained in 5% CO₂ incubator at 37°C.

Cell viability assay

HUVECs were seeded in 96-well plate at 1×10^4 cells/well. Cells were incubated in serum-deprived medium overnight, then exposed to Lipopolysaccharide (LPS, Sigma-Aldrich, 10 ng/mL) with or without Crocetin (1, 5, 10 ng/mL) for 24hr. Cell viability was measured by performing 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT, Sigma) assay as previously described[17].

Cell cytotoxicity assay

HUVECs were seeded in 12 well plate at 4×10^5 cells /well. Cells were incubated in serum-deprived medium overnight, then treated with LPS (10 ng/mL) with or without Crocetin (1, 5, 10 ng/mL) for 24hr. After then, supernatants from each condition were harvested. Cell toxicity was determined by the lactate dehydrogenase (LDH) leakage into supernatant using LDH assay kit (JianCheng Bio-Technology Co. China) in accordance with manufacturer's instructions.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

HUVECs were seeded in 6 well plate at 1.2×10^6 cells /well overnight, then treated with LPS (10 ng/mL) with or without Crocetin (1, 5, 10 ng/mL) for 24hr. After that, total RNA was isolated by ZR RNA

Fig. 1. Structural formula of Crocetin.

MiniPrep (Zymo Research) and reverse transcribed into cDNA by High capacity cDNA reverse transcription kit (Applied Biosystems) in accordance with manufacturer's instructions. Primers used for cDNA synthesis were as follow: MCP-1 (forward: 5'- AAG ATC TCA GTG CAG AGG CTC G-3'; reverse: 5'- CCA GGG GTA GAA CTG TGG TTC AA-3'); IL-8 (forward: 5'- TCT CAG CCC TCT TCA AAA ACT TCT C-3'; reverse: 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'); and housekeeping GAPDH (forward: 5'-CTC TCT GCT CCT CCT GTT CGA CAG-3'; reverse: 5'-GTG GAA TCA TAT TGG AAC ATG T-3'). PCR products were electrophoresed on 1.5% agarose gel containing 0.1 µg/mL dye (Gold view, SBS Genetech Co. China). Gels were visualized and imaged by a Gel-Doc image analyzer (Bio-Rad); bands were quantified by ImageJ program.

Determination of MCP-1 and IL-8 by enzyme-linked immune sorbent assay (ELISA)

HUVECs were treated as previously described in 2.4 section. Cell culture supernatants from each condition were collected, filtered through a 0.45µm cell Nalgene syringe filter (Thermo Scientific) to remove the debris. Then ELISAs for MCP-1 and IL-8 (Bender) were performed according to manufacturer's instruction.

Western blot

HUVECs were treated as previously described in 2.5 section. The whole cell lysate was extracted by RIPA lysis buffer system (Santa Cruz Biotechnology); to separate the protein from the cytoplasm and nucleus, HUVECs were treated with a Nucleus and Cytoplasm protein extracting kit (KeyGEN, China), according to manufacturer's instruction. Protein samples were equally loaded (30µg/lane) to NuPAGE 4-12% Bis-Tris Gel (Novex, Life technologies) and resolved by electrophoresis, then transferred to polyvinylidene difluoride (PVDF, Millipore) membrane. The membrane was further incubated with antibodies against IκBα, NF-κB p65 (Santa Cruz Biotechnology), or β-Actin (Sigma), followed by appropriate secondary antibodies incubation and ECL (Pierce) development. Immunoreactive bands were determined by Chemi-doc image analyzer (Bio-Rad), and further analyzed by ImageJ program.

Cell adhesion assay

HUVECs were treated as previously described in 2.4 section. U937 cells were labeled with 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM, 3µM final concentration, Beyotime, China) for 30min at 37 °C, re-suspended in serum-free medium. Labeled U937 cells (5×10⁴ cell/mL) were equally distributed into each well co-culture with HUVECs for 2hr at 37°C. After 2hr incubation, the supernatant was removed by gentle aspiration; wells were washed with warm PBS for 3 times. U937 cells bound to HUVECs were fixed with 4% paraformaldehyde in PBS, fluorescence was observed at 485nm excitation and 530nm emission wavelength. Images were further quantified and analyzed by ImageJ program.

Histological examination

Male Sprague-Dawley rats were kept under standard housing conditions. Rats received LPS (0.5 mg/kg) injection subcutaneously in the abdomen with or without oral administration of Crocetin (25, 50, 100 mg/kg) twice a day for consecutive three days. Animals were sacrificed 24hr after the last drug administration. Subcutaneous tissues were harvested, fixed in 10% buffered formalin and then embedded in paraffin for H&E staining.

Statistical analysis

Data were presented as mean ± S.E.M. One way ANOVA followed by Newman-Keuls test was applied for statistical analysis. A *p* < 0.05 * or *p* < 0.01 ** was considered as significant.

Results

LPS-induced cell death is alleviated by Crocetin treatment

HUVECs displayed decreased cell viability following 24hr LPS stimulation (Fig. 2), MTT assay was used to determine if Crocetin could alleviate this LPS-induced cell damage and cell death. Cell viability was normalized to the untreated group and expressed as the percentage (control group considered as 100% viability). As shown in Fig. 2A, elevated cell viability was achieved with a high dose Crocetin treatment ($p<0.05$) compared to Model (LPS only) group. Although no significant viability change was observed in Crocetin treatment alone at given doses (Fig. 2B).

Crocetin inhibits LPS-induced cytotoxicity

Lactate dehydrogenase (LDH), as an inflammatory biomarker, is prevalently used to evaluate cell inflammation and tissue damage. Our previous study suggested that Crocetin suppresses LPS-induced cell death, and in this study, we further investigated Crocetin's effect on decreasing LPS-induced cytotoxicity. HUVECs were treated for 24hr with LPS and Crocetin of different doses. LDH leakage level in the supernatant was elevated in Model (LPS only) group compared to untreated group ($p<0.05$). As expected, LDH elevation in the Model (LPS only) group was suppressed with Crocetin treatment in a dose-dependent manner (Fig. 3, $p<0.05$ or $p<0.01$), indicating the protective role of Crocetin on HUVECs inflammatory response.

Fig. 2. (A) Effect of Crocetin on cell viability. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin at concentration of 1, 5, 10 ng/mL for 24hr. The cell viability was measured by MTT assay. (B) Effect of Crocetin on cell viability. HUVECs were treated with Crocetin at different doses (0, 1, 5, 10 ng/mL) for 24hr. Cell viability was measured by MTT assay. Values were expressed as mean $\pm$ S.E.M from three independent experiments. * indicates $p<0.05$.

Fig. 3. Effect of Crocetin on LPS induced cytotoxicity. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin (1, 5, 10 ng/mL) for 24hr. Supernatants were collected for LDH leakage determination. Data were expressed as the mean $\pm$ S.E.M of the results from three independent experiments. * indicates $p<0.05$; ** indicates $p<0.01$.

Fig. 4. Crocetin's effect on MCP-1 and IL-8 mRNA expression levels. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin (1, 5, 10 ng/mL) for 24hr. The mRNA levels of MCP-1 and IL-8 were determined by RT-PCR. Data were expressed as the mean $\pm$ S.E.M of the results from three separate experiments. ** indicates $p < 0.01$.

Fig. 5. Crocetin's effect on MCP-1 (A) and IL-8 (B) secretions by ELISA. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin (1, 5, 10 ng/mL) for 24hr. Supernatants were collected to determine MCP-1 and IL-8 levels by ELISA. Data were expressed as the mean $\pm$ S.E.M of the results from three separate experiments. * indicates $p < 0.01$; ** indicates $p < 0.01$.

Elevated expression of chemokine MCP-1 and IL-8 is abrogated by Crocetin

MCP-1 and IL-8 are important pro-inflammatory chemokines involved in vascular inflammatory response and regulate immune cell migration to the lesion site. To understand Crocetin's role in inhibiting LPS induced MCP-1 and IL-8 expression and secretion, RT-PCR and ELISA were performed respectively. As in Fig. 4, MCP-1 and IL-8 gene expression levels were significantly increased with LPS stimulation. However, this elevation was abrogated by Crocetin treatment, in a dose dependent manner ($p < 0.01$). Furthermore, to confirm Crocetin's effect on MCP-1 and IL-8 secretions, the cell supernatants were collected for ELISA. In accordance with the RT-PCR results, MCP-1 (Fig. 5A) and IL-8 (Fig. 5B) secretions were both increased by LPS stimulation ($p < 0.01$) and subsequently decreased by Crocetin treatment at different doses as expected ($p < 0.05$ or $p < 0.01$).

Inflammatory cell adhesion and infiltration are alleviated by Crocetin treatment

In the aforementioned results, both chemokine MCP-1 and IL-8 levels were significantly elevated induced by LPS, while alleviated by Crocetin treatment. Based on the known role of MCP-1 and IL-8 in cell migration and adhesion, a functional assay was performed to investigate if Crocetin can block immune cell adhesion to the inflamed HUVECs. As indicated

Fig. 6. Crocetin's effect on U937 cell adhesion to HUVECs. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin (1, 5, 10 ng/mL) for 24hr; then co-cultured with fluorescently labelled U937 cells for 2hr, after which they were fixed and imaged by fluorescence microscopy. ** indicates $p < 0.01$.

Fig. 7. Crocetin's effect on immune cell adhesion and infiltration *in vivo*. Rats were received a subcutaneous injection on the abdomen with LPS (0.5 mg/kg) for 1 time and oral administration of Crocetin (25, 50, 100 mg/kg) twice a day for three days. 24hr after the last drug administration, rats were sacrificed. Subcutaneous tissues were harvested, fixed in 10% buffered formalin and embedded in paraffin for H&E staining.

in Fig. 6, U937, human leukemic monocyte lymphoma cells were fluorescently labeled, then co-cultured with HUVECs for 2hr at 37°C. In accordance with secreting MCP-1 and IL-8 level, LPS and Crocetin co-treated groups displayed a significantly lower number of adhered U937 ($p < 0.01$) compared to Model (LPS only) group, indicating U937 adhesion to inflamed HUVECs was abrogated. This result was further confirmed by H&E staining in murine subcutaneous endothelium in Fig. 7. LPS induced massive inflammatory cell infiltration with disarranged endothelium structure (Fig. 7B). However, Crocetin treatment markedly attenuated cell infiltration in a dose-dependent manner (Fig. 7C, D, and E). Moreover, the restored endothelial structure was observed in Crocetin treated groups (Fig. 7D and E).

Crocetin exerts its anti-inflammatory effect through suppression NF- κ B p65 transduction

Knowing that Crocetin downregulates LPS-induced MCP-1 and IL-8 expression, we next sought to study potential signaling pathway that Crocetin may affect. NF- κ B, known as a

Fig. 8. Effect of Crocetin on NF- κ B signaling inhibition. HUVECs were exposed to LPS (10 ng/mL) while treated with or without Crocetin (1, 5, 10 ng/mL) for 24hr. Cytosolic and nuclear fractions were prepared and resolved by SDS-PAGE. I κ B- α (A) NF- κ B p65 (B, cytosolic extraction; C, nuclear extraction) and were detected by western blot. β -Actin was used as loading control. Data were expressed as the mean $\pm$ S.E.M of three individual experiments. * indicates $p < 0.05$; ** indicates $p < 0.01$.

classic inflammation related signal pathway, is widely activated in inflammatory processes in different cell types. Upon NF- κ B signaling activation, I κ B kinase (IKK) is recruited to phosphorylate its substrate I κ B α . Associated with phosphorylation and degradation of I κ B α , the p65 subunit will be released from the I κ B α -p65-p50 complex, and translocate into the nucleus. As a result, the transcriptional activity of many pro-inflammatory cytokines and chemokines including IL-8 and MCP-1 will be triggered and elevated. According to our findings, LPS-induced I κ B α degradation was observed (Fig. 8A), associated with NF- κ B p65 translocation. This translocation manifests as decreased p65 level in the cytoplasm and concurrently increased level in the nucleus (Fig. 8B, C). However, Crocetin stabilizes I κ B α subunit and therefore, abrogates p65 translocation, inhibiting downstream pro-inflammatory cytokines and chemokines transcriptional activities.

Discussion

In this study, endotoxin Lipopolysaccharide (LPS) was used to induce the endothelial inflammatory response in HUVECs. As an exogenous stimulus, LPS has been widely used in endothelium damage and repair studies, due to its character of increasing cell permeability and impairing endothelium barrier, which is an essential step in inflammation initiation stage [18, 19]. This stress-induced cell death and cytotoxicity were determined by MTT assay (Fig. 2), and LDH leakage assay (Fig. 3), respectively. These indicators of inflammatory stress decreased under Crocetin treatment in a dose-dependent manner, indicating higher cell viability and lower LDH leakage. Also, our studies confirmed elevated levels of pro-inflammatory, and adhesion related chemokines MCP-1 and IL-8 under LPS stimulation in HUVECs. These induced effects were observed in both transcription and translation levels, which could be subsequently ameliorated by Crocetin treatment in a dose-dependent manner (Fig. 4, 5).

In CVDs such as atherosclerosis, inflammation is initiated by immune cell adhesion to endothelium [20], which may trigger a positive pro-inflammatory feedback loop, and further, amplify inflammation [21]. Thus, the inhibition of cell adhesion is promising for CVDs treatment. To explore this inhibition activity by Crocetin, a cell adhesion assay, which compares the physiological and pathological (inflammatory) vascular conditions [22, 23],

in MCP-1 and IL-8 was performed. The Crocetin-mediated inhibition was observed in monocyte adhesion to endothelial cells (Fig. 6). Moreover, Crocetin treatment was able to restore subcutaneous endothelium structure, as well as to alleviate immune cell infiltration to lesion site compared to the LPS treated group (Fig. 7).

Multiple pathways are proposed in the vascular injury and its inflammatory process according to growing evidence, including eNOS/PI3K/Akt [24], mitogen-activated protein kinase (MAPK) [25-27], transforming growth factor- β (TGF- β) [28], and NF- κ B signaling [29]. The inhibitory effect of Crocetin on MAPKs including ERK1/2, p38, and JNK has been widely studied and reported. However, Crocetin's effect on NF- κ B during vascular inflammation needs further investigation. As a key nuclear transcription factor, the NF- κ B pathway can be activated by factors such as bacterial endotoxin [30], inflammatory pharmacological agents, and T cell mitogens. Specifically, previous studies indicated it is mediated by upstream regulator toll-like receptor (TLR) family, especially TLR4. As a ligand of TLR[31], we applied LPS stimulation to activate I κ B α kinase (IKK) via tumor necrosis factor receptor (TNFR), and interleukin-1 receptor (IL-1R), which further phosphorylates and signals for degrading the cytoplasmic I κ B α subunit of the I κ B α -p65-p50 complex. Upon I κ B α degradation, p65 subunit is released and translocated into the nucleus, where it activates downstream inflammatory gene translational activity. Crocetin was observed to stabilize the I κ B α -p65-p50 complex, thereby blocking its signaling cascade (Fig. 8). These indicate that Crocetin achieves its anti-inflammatory activity via NF- κ B signaling pathway. This novel mechanism may contribute further understanding of NF- κ B's activation in cardiovascular disease, and opens up a new direction of Crocetin treatment in CVDs.

In conclusion, we demonstrated the efficacy of Crocetin in alleviating the inflammatory response stimulated by LPS in HUVECs. This stimulated inflammatory process is linked with CVD inflammation, which may also involve in diabetes, hypertension, and cancer [32-35]. Our study further supports Crocetin as a strong candidate anti-inflammatory drug with few side effects. Crocetin's potential clinical value may thus go beyond CVDs, and be applied in a wider range of inflammation related diseases.

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Disclosure Statement

Authors declare no conflict of interest.

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