

INHIBITION OF NF- κ B AND STAT3 BY QUERCETIN WITH SUPPRESSION OF ADHESION MOLECULE EXPRESSION IN VASCULAR ENDOTHELIAL CELLS

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Abstract

Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), P- and E-selectin play a key role for the initiation of vascular inflammation. In this study, we explored the mechanism by which quercetin may inhibit ICAM-1 and VCAM-1 expressions stimulated with lipopolysaccharide (LPS) in human umbilical vein endothelial cells (HUVEC). Quercetin prevented LPS-mediated increase of ICAM-1 and VCAM-1 expression. Stattic (6-Nitrobenzo[b]thiophene-1,1-dioxide), a small-molecule inhibitor of signal transducer and activator of transcription 3 (STAT3), inhibited both ICAM-1 and VCAM-1 expression stimulated with LPS. LPS induced I κ B α degradation within 1 hour. Quercetin did not affect the I κ B α degradation stimulated with LPS. However, in luciferase reporter assay, quercetin decreased the NF- κ B activity. On the other hand, quercetin prevented LPS-mediated increase of STAT3 phosphorylation. Quercetin reduced LPS-mediated THP-1 monocyte adhesion to HUVEC, in a concentration-dependent manner. These data provide a novel mechanism where quercetin inhibits NF- κ B and STAT3 activity resulting in suppression of ICAM-1 and VCAM-1 expressions in the vascular wall.

Rezumat

Moleculele de adeziune intracelulară (ICAM-1), moleculele de adeziune vasculară (VCAM-1), selectina P și selectina E prezintă un rol definitoriu în etapa inițierii procesului inflamator. În studiul prezentat, am investigat mecanismul prin care quercetina poate inhiba exprimarea acestor markeri prin intermediul lipopolizaharidelor (LPS) în celulele endoteliale ale venei ombilicale umane (HUVEC). Quercetina a inhibat expresia ICAM-1 și VCAM-1 mediată de LPS. O moleculă cu rol de inhibiție a semnalului traductor sau activator pentru transcriere, STAT3 (moleculă de semnalizare și de activare al factorului de transcripție 3) a inhibat exprimarea ambelor tipuri de molecule de adeziune, stimulată prin lipopolizaharide. Acestea au indus degradarea I κ B α (I κ B α) într-o oră. Quercetina nu a interferat cu degradarea LPS-indusă a I κ B α . Cu toate acestea, în studiile cu luciferază, quercetina a modificat activitatea NF- κ B. Pe de altă parte, quercetina a inhibat fosforilarea STAT3 mediată de LPS, a redus adeziunea monocitelor THP-1 mediată de LPS, în celulele HUVEC, dependent de concentrație. Aceste date experimentale explică un mecanism nou, prin care quercetina inhibă activitatea NF- κ B și STAT3, ceea ce are drept consecință inhibarea expresiei ICAM-1 și VCAM-1 în peretele vascular.

Keywords: quercetin, signal transducer and activator of transcription 3, Intercellular adhesion molecule 1, vascular cell adhesion molecule 1

Introduction

The activation of vascular endothelial cells is an important event from the beginning of the blood vessel inflammation. Leukocytes are in contact with the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expressed on endothelial cells, and this is an important event causing vascular inflammation [1, 2]. Quercetin, a dietary flavonol, is known for its effect on preventing and treating various cardiovascular diseases. Quercetin reduces the inflammation-induced over-expression of VCAM-1 and ICAM-1's protein and transcript in human umbilical vein endothelial cell (HUVEC) [3]. In cultured human

endothelial cells, quercetin also reduces the cytokine-induced cell-surface expression of VCAM-1 and E-selectin [4]. Moreover, flavonols significantly reduce the endothelial expression and release of macrophage colony stimulating factor (M-CSF). The decrease in endothelial inflammatory gene expression was related to the inhibition of NF- κ B and activation of activator protein 1 (AP-1) but not to intracellular oxidative stress [5]. Nuclear factor kappaB (NF- κ B) and AP-1 binding activity was inhibited by quercetin resulting in the inhibition of expression of VCAM-1 and ICAM-1 [6].

On the other hand, signal transducer and activator of transcription 3 (STAT3) is also known, in addition to

NF- κ B, as an important transcription factor involved in regulating inflammation. The Janus kinase (JAK)/STAT3 pathway may be important in chronic inflammatory control [7]. Quercetin ameliorates allergic encephalomyelitis by blocking interleukin (IL)-12 signalling through JAK-STAT pathway in T lymphocytes [8]. Quercetin reduces STAT3 activation by IL-6 and also modulated the expression of two target genes regulated by STAT3, cyclin D1 and matrix metalloproteinase-2 (MMP-2) [9]. However, the involvement of STAT3 is not known for the regulation of leukocyte adhesion molecule expression by quercetin in vascular endothelial cells. Therefore, the purpose of this study was to investigate the involvement of STAT3 with respect to the regulation of ICAM-1 and VCAM-1 expression by quercetin in HUVEC.

Materials and Methods

Endothelial cell basal medium (EBM)-2 Bullet kit was obtained from Lonza (USA). RPMI 1640 medium, foetal bovine serum (FBS), penicillin-streptomycin, phosphate buffer saline (PBS), trypsin-EDTA were obtained from Invitrogen (USA). Lipopolysaccharide (LPS) was obtained from List Biological Laboratories (USA). Gelatin, Calcein-AM, JSH-23 (4-Methyl-*N*¹-(3-phenyl-propyl)-benzene-1,2-diamine) and quercetin were obtained from Sigma-Aldrich (USA). Anti-bodies against human vascular cell adhesion molecule-1 (VCAM-1), human intercellular adhesion molecule-1 (ICAM-1), I κ B α , β -tubulin and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA).

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (USA) at passage 1 and maintained in EBM-2 Bullet kit growth medium at 37°C in a humidified atmosphere of 5% CO₂. In all experiments, cells were used at passage 4-9. HUVECs were plated at 90-95% confluence treated for 1 h with 1, 10, and 20 μ M of quercetin prior to LPS (1 μ g/mL) stimulation for 1 or 8 h. A group of cells treated only with dimethylsulfoxide (DMSO) was used as a solvent control. Human-derived THP-1 macrophage cell lines (a human monocytic cell line derived from an acute monocytic leukemia patient) were obtained from the American Type Culture Collection (ATCC) (USA). THP-1 cells were cultured in RPMI 1640, and supplemented with 2 mM L-lutamine, 100 μ g/mL streptomycin, 100 IU/mL penicillin and 10% FBS.

The endothelial cells were pre-treated with drug prior to LPS stimulation. After treatment, the cells were washed twice with PBS. Whole cell lysates were prepared Radioimmunoprecipitation assay buffer (RIPA buffer) containing the Protease Inhibitor Cocktail V (Switzerland). Protein concentration

was determined by using the Bio-Rad protein assay (Bio-Rad Lab, USA) with bovine serum albumin (BSA) as the standard. The lysates were resolved on 8% or 10% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to an Immobilon-P membrane (Millipore, USA) and the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature 1 h. The membrane was incubated for overnight with primary antibodies of anti-human ICAM-1, anti-human VCAM-1 and I κ B α . After three washes with TTBS buffer, the membrane was then incubated for 1 h with HRP-conjugated secondary antibodies. The levels of ICAM-1, VCAM-1 and I κ B α proteins were determined by using an Enhanced Chemoluminescence Plus kit (Amersham Biosciences, USA) and Fujifilm LAS-3000 system (Fujifilm, Japan). Anti-human β -tubulin antibody was used for the loading control. Each image of Western blot was quantified with Multi Gauge software version 2.3 (Fujifilm, Japan). An NF- κ B reporter plasmid, which contains 5 $\times$ NF- κ B response elements fused to luciferase, was purchased from Stratagene (La Jolla, CA, USA). HUVECs were co-transfected with 3 μ g of reporter gene and plasmid cytomegalovirus- β -gal, using the Lipofectamine (Invitrogen, Carlsbad, CA, USA) method. After transfection, cells were treated for 1 h with quercetin (1-20 μ M) and JSH (50 μ M) prior to LPS (1 μ g/mL) stimulation for 16 h, and then lysed to determine luciferase and β -galactosidase (β -gal) activities. Luciferase activity was analysed using a Lumat LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany), and a β -gal assay was performed to normalize transfection efficiency.

HUVECs were grown in EBM-2 Bullet kit growth medium at a density of 2.0×10^5 cells/well on 24-well plates. Endothelial cells at 90 - 95% confluence were treatment with quercetin 20 μ M for 1 h prior to 1 μ g/mL of LPS stimulation for 8 h. THP-1 cells were labelled with Calcein-AM (5 μ M) in RPMI 1640 medium containing 10% FBS for 30 min. After two times of extensive washing with PBS, the labelled THP-1 cells were seeded at a density of 5.0×10^5 cells/well onto endothelial cells which were treated with the quercetin and/or LPS and, then incubated for 1 h at 37°C while gentle shaking. After incubation, non-adherent cells were removed by gentle washing two times with PBS. Images were registered at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope.

The results are presented as means $\pm$ SEM for each treatment group in each experiment. Data were analysed by one-way ANOVA followed by Scheffe *post-hoc* test for multiple comparisons. Statistical

software SPSS (USA) was used in. $p < 0.05$ were considered statistically significant.

Results and Discussion

Treatment of endothelial cells with LPS (1 $\mu\text{g/mL}$) increased ICAM-1 and VCAM-1 expression. 8 hours after LPS treatment, maximal expression of ICAM-1 and VCAM-1 was attained (data not shown). To determine whether LPS-stimulated ICAM-1 and VCAM-1 expression is affected by quercetin, endothelial cells were treated for 1 h with quercetin (1–20 μM) prior to LPS (1 $\mu\text{g/mL}$) stimulation for 8 h. Quercetin inhibited ICAM-1 and VCAM-1 expression stimulated with LPS in a concentration-dependent manner, significantly (Figure 1).

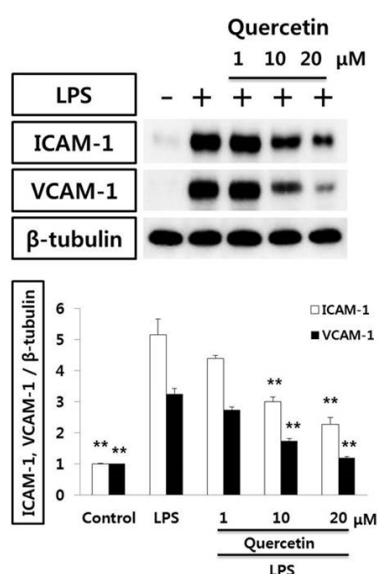


Figure 1.

Effect of quercetin on the protein expression level of adhesion molecule in HUVEC stimulated with LPS. Endothelial cells were treated with 1, 10, 20 $\mu\text{mol/L}$ of quercetin for 1 h prior to LPS (1 $\mu\text{g/mL}$) stimulation for 8 h. (A) Effect of quercetin on the protein expression level of ICAM-1 and VCAM-1 stimulated with LPS in HUVEC. Cell extracts were resolved on 8% SDS-polyacrylamide gel and Western blot analysis with the respective primary antibody against ICAM-1 and VCAM-1. β -actin was used as an internal control. The bar graph represents the amount of ICAM-1 and VCAM-1 estimated by image scanning and is expressed in arbitrary units. Values are means $\pm$ SEM of 3 independent experiments. Statistical significance assessed by one-way ANOVA followed by Scheffé *post-hoc* test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$ vs. LPS).

To determine whether LPS-stimulated ICAM-1 and VCAM-1 expression is affected by certain signalling pathways, endothelial cells were treated for 1 h with 50 μM of signalling pathway inhibitor prior to LPS (1 $\mu\text{g/mL}$) stimulation for 8 h. JSH-23,

an inhibitor of NF- κB transcriptional activity, inhibited VCAM-1, but not ICAM-1 expression stimulated with LPS, significantly. Stattic, a small-molecule inhibitor of STAT3 activation and dimerization, inhibited neither ICAM-1, nor VCAM-1 expression stimulated with LPS, significantly. PD98059, a selective and reversible inhibitor of mitogen-activated protein kinase (MAPK)-activating enzyme, did not inhibit neither ICAM-1 nor VCAM-1 expression stimulated with LPS. SB202190, a selective inhibitor of p38 MAPK, inhibited VCAM-1 expression but not ICAM-1 expression stimulated with LPS, significantly (Figure 2).

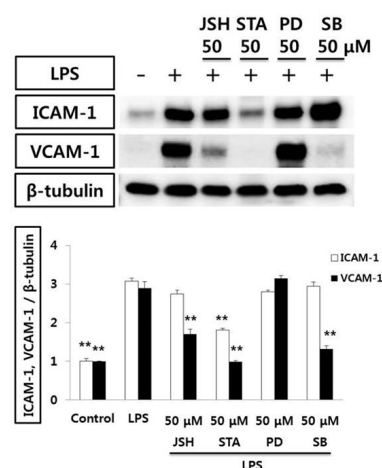


Figure 2.

Effect of inhibitors of signalling pathway on the protein expression level of adhesion molecule stimulated with LPS in HUVEC. Endothelial cells were treated with 50 $\mu\text{mol/L}$ of JSH-23 (NF- κB inhibitor), Stattic (STAT3 inhibitor), PD 98059 (a selective and reversible inhibitor of MAPK-activating enzyme), SB 202190 (p38 MAPK inhibitor) for 1 h prior to LPS (1 $\mu\text{g/mL}$) stimulation for 2 h, respectively. Cell extracts were resolved on 8% SDS-polyacrylamide gel and Western blot analysis with the respective primary antibody against ICAM-1 and VCAM-1. β -actin was used as an internal control. The bar graph represents the amount of ICAM-1 and VCAM-1 estimated by image scanning and is expressed in arbitrary units. Values are means $\pm$ SEM of 3 independent experiments. Statistical significance assessed by one-way ANOVA followed by Scheffé *post-hoc* test for multiple comparisons (** $p < 0.01$ vs. LPS).

Treatment of endothelial cells with LPS (1 $\mu\text{g/mL}$) increased I κ B α degradation. 1 h after LPS treatment, a significant decrease of I κ B α was attained (Figure 3A). To determine whether LPS-induced I κ B α degradation was affected by quercetin, endothelial cells were treated for 1 h with quercetin (1–20 μM) prior to LPS (1 $\mu\text{g/mL}$) stimulation for 1 h. Quercetin did not prevent LPS-induced I κ B α degradation. On the other hand, the treatment of endothelial cells with

LPS (1 $\mu\text{g/mL}$) increased STAT3 phosphorylation. 2 h after the treatment, the maximal phosphorylation of STAT3 was attained. To determine whether LPS-stimulated STAT3 phosphorylation is affected by quercetin, endothelial cells were treated for 1 h with quercetin (1 - 20 μM) prior to LPS (1 $\mu\text{g/mL}$) stimulation. Quercetin inhibited STAT3 phosphorylation stimulated with LPS in a concentration-dependent manner, significantly (Figure 3B).

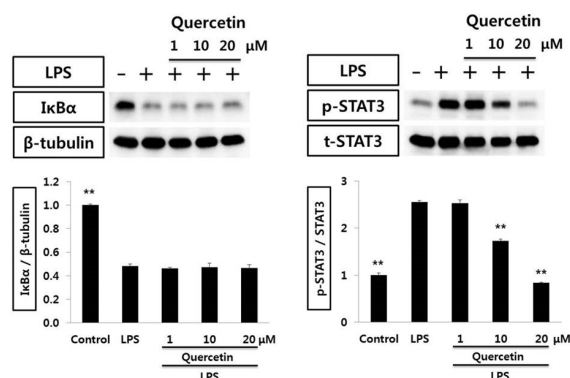


Figure 3.

Effect of quercetin on the protein expression level of IκBα (A) and the phosphorylation level of STAT3 (B) in HUVEC stimulated with LPS. Endothelial cells were treated with 1 - 20 $\mu\text{mol/L}$ of quercetin for 1 h prior to LPS (1 $\mu\text{g/mL}$) stimulation for 2 h. Cell extracts were resolved on 8% SDS-polyacrylamide gel and Western blot analysis with the respective primary antibody against IκBα and phospho-STAT3. β-actin was used as an internal control. The bar graph represents the amount of IκBα or phospho-STAT3 estimated by image scanning and is expressed in arbitrary units. Values are means $\pm$ SEM of 3 independent experiments. Statistical significance assessed by one-way ANOVA followed by Scheffe *post-hoc* test for multiple comparisons (* $p < 0.01$ vs. LPS).

Treatment of endothelial cells with LPS (1 $\mu\text{g/mL}$) increased NF-κB luciferase activity compared with control, significantly (Figure 4). To determine whether LPS-induced NF-κB luciferase activity was affected by quercetin, endothelial cells were treated for 1 h with quercetin (1 - 20 μM) prior to LPS (1 $\mu\text{g/mL}$) stimulation for 1 h. Quercetin decreased LPS-induced NF-κB luciferase activity, significantly (Figure 4). Moreover, JSH (50 μM), an inhibitor of NF-κB, significantly attenuated the NF-κB luciferase activity stimulated with LPS (Figure 4). The adhesion of THP-1 cells to endothelial cells was measured using the quantitative monolayer adhesion assay. The adhesion of THP-1 cells onto endothelial cells increased by five folds by LPS (1 $\mu\text{g/mL}$) stimulation for 8 h. Quercetin (20 μM) inhibited the adhesion of THP-1 cells to endothelial cells stimulated with LPS, in a concentration-dependent manner (Figure 5).

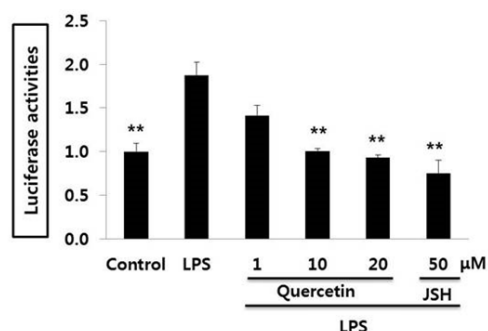


Figure 4.

Effect of quercetin on the NF-κB luciferase assay stimulated with LPS. An NF-κB reporter plasmid, which contains 5 $\times$ NF-κB response elements fused to luciferase, was purchased from Stratagene (La Jolla, CA, USA). HUVECs were co-transfected with 3 μg of reporter gene and plasmid cytomegalovirus-β-gal, using the Lipofectamine (Invitrogen, Carlsbad, CA, USA) method. After transfection, cells were treated for 1 h with quercetin (1-20 μM) and JSH (50 μM) prior to LPS (1 $\mu\text{g/mL}$) stimulation for 16 h, and then lysed to determine luciferase and β-galactosidase (β-gal) activities. Luciferase activity was analysed using a Lumat LB960 luminometer (Berthold Tech., Bad Wildbad, Germany), and a β-gal assay was performed to normalize transfection efficiency.

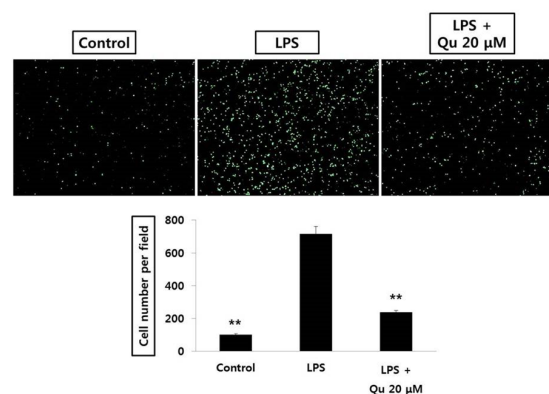


Figure 5.

Effect of quercetin on THP-1 monocyte adhesion to LPS in HUVEC stimulated with LPS. Endothelial cell was treated with 20 μM of quercetin prior to LPS (1 $\mu\text{g/mL}$) stimulation for 8 h. THP-1 cells were labelled with Calcein-AM (5 μM) for 30 min. The labelled THP-1 cells were seeded at a density of 5.0×10^5 cells/well onto endothelial cells treated with the inhibitor and/or LPS and then incubated for 1 h. Microphotographs (four independent experiments) were obtained using fluorescence microscopy, Magnification $\times 100$. The bar graphs represent the cell number of THP-1 monocyte. Values are means $\pm$ SEM of 3 independent experiments. Statistical significance assessed by one-way ANOVA followed by Scheffe *post-hoc* test for multiple comparisons (** $p < 0.01$ vs. LPS).

In this study, quercetin significantly inhibited the expression of leukocyte adhesion molecule such as ICAM-1 and VCAM-1 stimulated with LPS. These results are in agreement with already reported results [3, 4]. Contact of leukocytes with endothelial cells is a critical event for inducing vascular inflammation. Given these results, quercetin proves to be the phytochemical that interferes with the initial phase of vascular inflammation.

Phytochemicals may have anti-inflammatory effects by inhibiting the IKK/NF- κ B or STAT3 pathway. Quercetin has been reported to inhibit NF- κ B activation, the driving force for inflammation [10]. I κ B dissociation from RelA/p65-p50 complex is crucial for NF- κ B activity [11]. In this study, quercetin did not have any effect on I κ B α degradation. However, quercetin significantly inhibited NF- κ B reporter gene activity in luciferase reporter assay, in a concentration - dependent manner. On the other hand, JSH-23, an inhibitor of NF- κ B transcriptional activity, significantly inhibited the expression of VCAM-1, but not ICAM-1 stimulated with LPS. SB 202190, a selective inhibitor of p38 mitogen-activated protein kinase (MAPK), significantly inhibited the expression of VCAM-1, but not ICAM-1 stimulated with LPS. PD98059, a highly selective inhibitor of MEK (MAPK kinase) 1, did not inhibit the expression of both VCAM-1 and ICAM-1 stimulated with LPS.

Given these results, quercetin seems to attenuate NF- κ B activity which is required for the expression of VCAM-1, but not ICAM-1 in vascular endothelial cells. Moreover, due to the activation of p38 MAPK, but not MEK by LPS, the expression of VCAM-1 may be increased.

On the other hand, because ICAM-1 is the target of STAT3 [12], quercetin was investigated whether the expression of ICAM-1 is inhibited by an intervention of STAT3 activity. Quercetin significantly inhibited STAT3 phosphorylation stimulated with LPS, in a concentration - dependent manner. On the other hand, Stattic, a small-molecule inhibitor of STAT3 activation and dimerization, significantly inhibited the expression of VCAM-1 as well as ICAM-1 stimulated with LPS. Given these results, quercetin seems to attenuate STAT3 activity, which is required for the expression of VCAM-1 as well as ICAM-1 in vascular endothelial cells. However, quercetin showed weaker or no inhibitory effect on IL-6-induced STAT3 phosphorylation in endothelial cells [13]. Therefore, the effect of quercetin on the STAT3 activity, with respect to vascular inflammation may vary from the external ligand.

On the other hand, other mechanisms of quercetin may include nuclear erythroid 2-related factor (Nrf2). Nrf2 is an important transcription factor in controlling the anti-oxidative system [14]. Therefore,

further studies remain to clarify the effect of quercetin on Nrf2 activity, which may result in attenuation of expression of ICAM-1 and VCAM-1 in vascular endothelial cells.

THP-1 is a human leukaemia monocytic cell line, which has been extensively used to study monocyte/macrophage functions mechanisms, signalling pathways, and nutrient and drug transport [15]. The increase of THP-monocyte adhesion to endothelial cells by LPS supports the crucial role of ICAM-1 and VCAM-1 in the early phase of vascular inflammation [16]. Therefore, whether quercetin prevents the THP-1 monocyte adhesion to endothelial cells was studied. In this study, quercetin significantly reduced the adhesion of THP-1 monocyte to endothelial cell.

Conclusions

Quercetin reduces the expression of leukocyte adhesion molecule such as ICAM-1 and VCAM-1 onto vascular wall through an inhibition of STAT3 as well as NF- κ B activity. This effect may be beneficial for an early prevention of vascular inflammatory disease such as arteriosclerosis in humans.

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