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RESEARCH ARTICLE

The anti-HSV-1 effect of quercetin is dependent on the suppression of TLR-3 in Raw 264.7 cells

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Abstract Quercetin is a major component of the plant *Glycyrrhiza uralensis*, which is largely used as a traditional medicine in Asia. Quercetin has been reported to have several biological activities, which include anti-viral and anti-inflammatory effects. We explored the molecular mechanism linking anti-viral and anti-inflammatory activities using an in vitro herpes simplex virus-1 (HSV-1) infection model. Raw 264.7 cells were infected with HSV-1 in the presence or absence of different concentrations of quercetin and infected cell lysates were harvested 24 h later. HSV plaque reduction assays, western blotting (HSV-1gD, HSV-1 ICP0, TLR-2, 3, 9, NF-κB, IRF3), and real time PCR (HSV-1ICP0, HSV-1UL13, HSV-1UL52) were performed to elucidate the mechanism responsible for the anti-HSV-1 effect of quercetin. In addition, TNF-α level was measured. Quercetin significantly lowered HSV infectivity in Raw 264.7 cells and inhibited the expressions of HSV proteins (gD, ICP0) and genes (ICP0, UL13, UL52). Interestingly, quercetin specifically suppressed the expression of TLR-3, and this led to the inhibitions of inflammatory transcriptional factors (NF-κB and IRF3). These findings suggest that the anti-HSV-1 effects of quercetin are related to the suppression of TLR-3 dependent inflammatory responses in Raw 264.7 cells.

Keywords Quercetin · HSV-1 · Raw 264.7 cells · TLR-3 · Anti-inflammation

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Introduction

Quercetin is one of the major flavonoids derived from licorice and is also present in a variety of fruits, vegetables, and herbs (Russo et al. 2012). Quercetin has been shown to have diverse pharmacological effects, which include anti-inflammatory, anti-cancer, and anti-viral effects (Cai et al. 2013; Kim et al. 2015). Many studies have shown that quercetin exerts a strong inhibitory effect on inflammatory responses, which suggests quercetin is a promising anti-inflammatory agent (Chang et al. 2013; Lee et al. 2013; Liao and Lin 2014; Takashima et al. 2014; Zou et al. 2015). Zou et al. demonstrated that quercetin could suppress lipopolysaccharide-induced inflammation (Zou et al. 2015), and Takashima et al. showed that intratracheally administered quercetin protected against lipopolysaccharide-induced acute lung injury (Takashima et al. 2014).

Quercetin has also been reported to have anti-viral effects against Epstein-Barr virus (EBV), influenza virus, hepatitis B virus (HBV), hepatitis C virus (HCV), and herpes simplex virus (HSV) (Yu et al. 2007; Cheng et al. 2015; Lee et al. 2015; Lu et al. 2015; Wu et al. 2015). Quercetin inhibited influenza virus infection in a wide range of strains by interacting with HA2 subunits and

suppressed HBV replication by reducing heat shock protein levels and HBV transcription levels (Cheng et al. 2015; Wu et al. 2015). Quercetin also appeared to have a strong antiviral activity in chronic HCV infection (Lu et al. 2015). In addition, quercetin and its analogs were found to robustly inhibit HIV-1 reverse transcriptase (Yu et al. 2007).

Various types of quercetin extracts have been reported to have anti-herpetic effects (Chen et al. 2011; Cho et al. 2015; Hung et al. 2015). In particular, Hung et al. found that quercetin inhibited HSV-1 viral entry in a manner that seemed to be associated with down-regulation of NF-κB expression in Vero cells (Hung et al. 2015). NF-κB is a universal transcription factor and plays a central role in inflammatory processes. However, the molecular mechanistic links between anti-HSV activity and anti-inflammatory effects have not been explored, and it has not been determined whether the anti-HSV effect of quercetin is due to the targeting of viruses and/or its effects of host cellular components.

Host immune system primarily recognizes HSV infection using Toll like receptors (TLRs), which stimulate various anti-viral mechanisms including Type I IFN (IFN-α, IFN-β) and pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) responses (Egan et al. 2013; Achek et al. 2016). Signaling through TLRs can activate two different downstream pathways: the MyD88- and TRIF-dependent pathways. MyD88 is a common downstream effector and induces the activation of NFκB (Takeda et al. 2003), whereas TRIF is responsible for regulating MyD88-independent pathways, which stimulate IRF3 and hence the expressions of type I IFNs genes (Takeda et al. 2003). However, excessive TLR-mediated cellular responses can cause chronic inflammation, which sometimes results in inflammatory or infectious disease. Therefore, modulation of TLR responses might prove to be a useful, and novel anti-inflammatory strategy (O'Neill 2003).

HSV-1 is known to recognize the human TLRs: TLR2, TLR3, and TLR9 (Krug et al. 2004; Kurt-Jones et al. 2004; Zhang et al. 2007). TLR3 is considered the principal mediator of viral recognition because it recognizes double-stranded RNA produced during viral replication, and interestingly, TLR3-dependent Type I IFNs were found to be critical for major immune responses to HSV-1 infection in the central nervous system (Zhang et al. 2007). TLR9 is stimulated by non-methylated double-stranded (ds) CpG-rich DNA, and thus, recognizes HSV infection (Krug et al. 2004). Interestingly, the TLR2-induced inflammatory response in HSV-1 infections appears to damage host cells, which suggests that in some contexts HSV-1- induced TLR responses could trigger pathological rather than protective outcomes (Kurt-Jones et al. 2004). As mentioned above, quercetin inhibits the activation of NF-κB, which is induced by bacterial and viral infections. However, whether quercetin can regulate HSV-1- induced TLRs responses or modulates IRF3 signaling has not been determined.

In this study, we investigated the inhibitory effect of quercetin on HSV-1 infection, and sought to determine whether quercetin can modulate inflammatory genes (NF-κB, IRF-3) and associated upstream pathways (TLRs) in HSV-1 infected Raw 264.7 cells.

Materials and methods

Preparation of quercetin

Quercetin was obtained from Sigma-Aldrich Co. (USA), dissolved in DMSO, filtered at 0.22-μm, and stored at −20 °C until use.

Virus and cell culture

Raw 264.7 cells and Vero cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA), 100U/ml penicillin and streptomycin (Gibco, USA) at 37 °C in a 5% CO_2 humidified atmosphere. HSV-1 stocks were propagated in Vero cells, which were then aliquoted and stored at −80 °C.

Plaque reduction assay

To assess the anti-HSV-1 effect of quercetin, Raw 264.7 cells were cultured in six-well culture plates (1×10^6 cells/well) and infected with HSV-1 at 0.1 MOI (multiplicity of infection) in the presence or absence of quercetin for 24 h. Cells were then lysed using five freeze/thaw cycles. Vero cells (3×10^5 cells/well) were seeded in 12-well culture plates for 24 h and incubated with cell lysates at 37 °C for 1 h. Inoculums were removed and overlaid with 0.5% methylcellulose-DMEM medium, and 4 days later, cells were stained with crystal violet in 50% methanol and plaques were confirmed.

Western blot analysis

Raw 264.7 cells were infected with HSV in the presence or absence of quercetin for 24 h. Proteins in cell lysates were quantified by the Bradford assay, separated by electrophoresis, and transferred to nitrocellulose membranes, which were then incubated with 1st and 2nd antibodies. Blots were visualized by enhanced chemiluminescent (ECL) detection solutions (DoGEN). The 1st antibodies used were anti-HSV1 ICP0 Ab (Abcam, USA), anti-HSV gD(Abcam, USA), anti-NFκB Ab (Milipore, USA), anti-IRF3 Ab (Cell

signaling, USA), anti-TLR-2, TLR- 3, TLR-9 Abs (Cell signaling, USA), and anti-β-actin Ab (Sigma, USA).

Quantitative RT-PCR

RNA was extracted from HSV infected Raw 264.7 cells treated with or without quercetin using an RNeasy Mini kit (Qiagen, USA), and synthesized into cDNA using M-MLV Reverse Transcriptase (m/biotech, USA). The resulting cDNA was analyzed for the expressions of HSV-1 genes by qRT-PCR. Gene expression levels were determined using SYBR Green reagent (BIOLINE) and a Step One Plus™ Real-time PCR system (Applied Biosystems, USA). 18sRNA was used as an internal control. PCR primer sequences were as follows: ICP0, ICP0F (5′-CTGTCGCCTTACGTGAACAA-3′)/ICP0R (5′-CCATGT TTCCCGTCTGGTC-3′); HSV-1 gD, gDF (5′- ACGAC-CAACTACCCCGATCA -3′)/gDR (5′- GAGGCATC-CACCAAGGCATA -3′); UL52 and UL13, UL52F (5′-GACCGACGGGTGCGTTAT T-3′)/UL52R (5′-GAAG-GAGTCGCCATTTAGCC-3′) and UL13F (5′-GCGACCT GCTGGTCATGTG-3′)/UL13R (5′-TGCGAGCCAATCC TTGAAG-3′), respectively.

ELISA assay

To analyze the anti-inflammatory effects of quercetin, ELISA was used to assess the expression of TNF-α in HSV infected Raw 264.7 cells. Briefly, Raw 264.7 cells were seeded in 24-well plates and infected with HSV-1 in presence or absence of quercetin. Cell free supernatants were harvested 24 h later to assess cytokine production using a mouse enzyme-linked immunosorbent assay kit (BD, USA). Absorbance was measured at 450 nm using a microplate reader (BMG Labtech, Germany).

Statistical Analysis

Data were processed using Microsoft Excel and results are presented as means ± SDs. Comparisons of several means were performed by one-way or two-way analysis of variance followed by Fisher's exact test to identify significant differences between groups. *p*- values of less than 0.05 were considered significant.

Results

Effect of quercetin on the infection of Raw 264.7 cells by HSV-1

To evaluate the anti-HSV-1 effect of quercetin, Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in presence or absence of quercetin (10, 20, 30 μg/ml). In another set of experiment, Raw 264.7 cells were first infected with HSV-1 at 0.1 MOI and 2 h later, quercetin (10, 20, 30 μg/ml) was added. After 24 h, infected cell lysates were subjected to five freeze/thaw cycles to release HSV-1 virions. Subsequently, virion-containing cell lysates were incubated with Vero cells and infectivity was assessed based on plaque formation. Figure 1b, c show a dramatic decrease in plaque formation in Vero cells when they were incubated with infected cell lysates treated with quercetin (50% decrease for 10 μg/ml quercetin, 80% decrease for 20 μg/ml quercetin, and 90% decrease for 30 μg/ml of quercetin). In addition, Fig. 1d, e present a similar decrease in plaque formation from infected cell lysates with post- treatment with quercetin. We also confirmed that quercetin was not cytotoxic to Raw 264.7 cells using a CCK-8 assay (CC50 was greater than 50 μg/ml, data not shown). These results show quercetin dose-dependently suppressed HSV-1 infection in Raw 264.7 cells.

Effect of quercetin on HSV-1 gD and ICP0 protein levels

To determine whether quercetin inhibits the expression of HSV-1 viral proteins, Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) and western blotted for HSV-1 gD and ICP0 proteins. HSV-1 glycoprotein D (gD) is known to be required for viral entry into host cells and ICP0 is one of the first genes to be expressed in the HSV-1 replication cycle. Figure 2a–c show that quercetin significantly reduced HSV-1 gD and ICP0 protein levels in HSV-1 infected Raw 264.7 cells. These findings suggest quercetin targets the viral life cycle at viral entry and replication.

Effect of quercetin on the gene expressions of HSV-1 ICP0, UL13, and UL52

Next, we examined whether quercetin suppresses HSV-1 replication by regulating the expressions of replication-associated viral genes. After viral entry into the host, several HSV-1 viral proteins, encoded by genes called immediate-early (IE), early (E), and late (L) genes, are serially produced. ICP0, UL13 and UL52 belong to IE, L, and E genes, respectively, and are essential for HSV replication. Therefore, Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20 μg/ml) for 24 h. RNAs were then extracted and HSV-1 ICP0, UL13 and UL52 gene expressions were analyzed by real time qPCR. As shown in Fig. 3a and b, the expression levels of ICP0, UL13, and UL52 were significantly and dose-dependently inhibited by quercetin. These

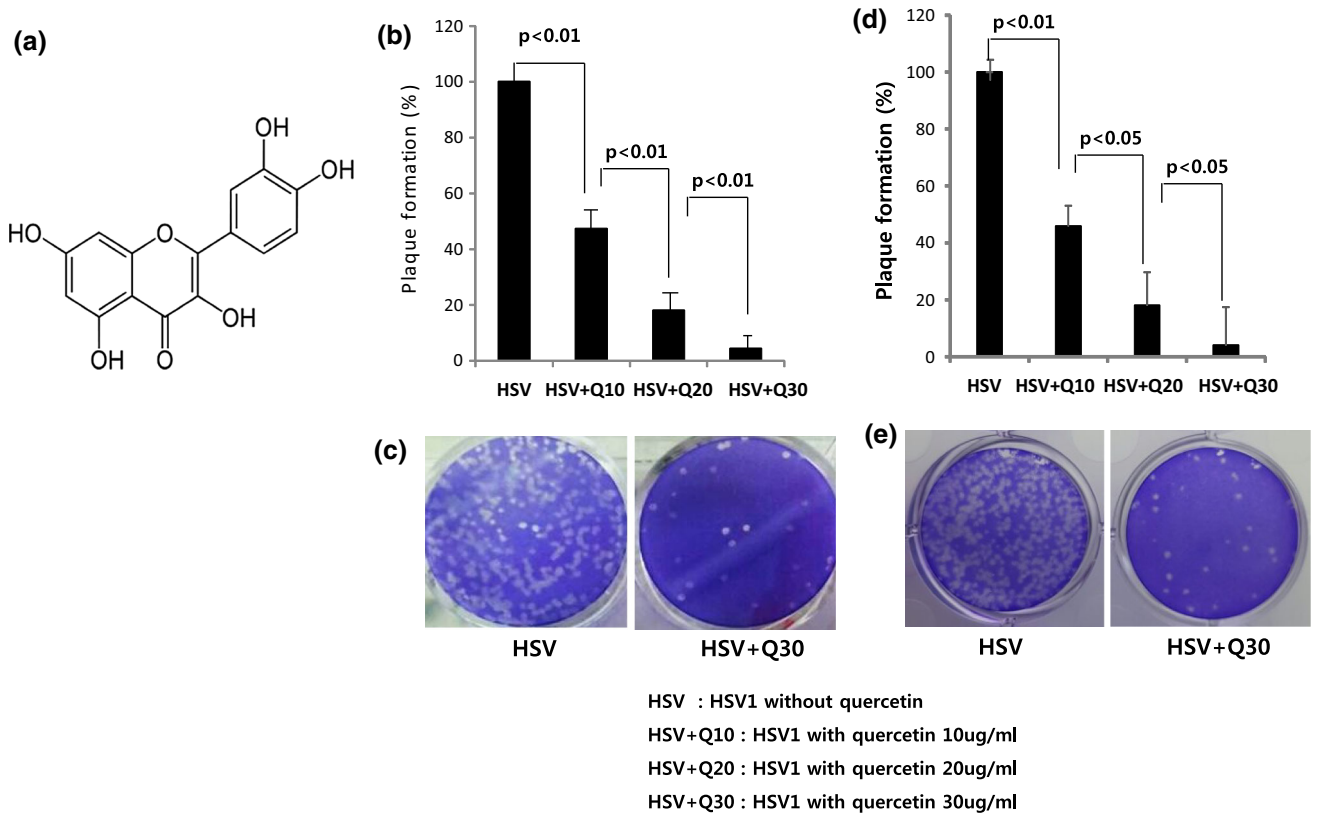

Fig. 1 Effect of quercetin on the infectivity of HSV-1 in Raw264.7 cells. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI (multiplicity of infection) in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) for (**b**) and (**c**). Also, Raw 264.7 cells were infected with HSV-1 at 0.1 MOI and quercetin (0, 10, 20, 30 μg/ml) was added at 2 h post-infection for (**d**) and (**e**). Infected Raw 264.7 cell lysates were harvested 24 h later, and then Vero cells were incubated in the cell lysates. The inhibitory effect of quercetin on HSV infection was determined by a plaque reduction assay. **a** The structure of quercetin, **b**, **d** plaque formation (%) in Vero cells, and **c**, **e** representative plaque formation in Vero cells incubated with infected cell lysates

observations suggest that quercetin suppresses the expression of several viral genes by inhibiting the immediate early expression of viral ICP0.

Effect of quercetin on HSV-1-induced NF-κB and IRF-3 protein levels

HSV-1 infection is known to induce inflammatory responses via host cell transcription factors, NF-kB and IRF3. Therefore, we examined whether quercetin could modulate the HSV-1-induced activations of NF-κB or IRF3. Quercetin was found to suppress NF-κB markedly in HSV-1 infected cells (Fig. 4a), and interestingly, at 30 μg/ml reduced IRF3 levels to almost zero (Fig. 4a). Furthermore, quercetin dose-dependently reduced HSV-1-induced activations of NF-κB and IRF-3 (Fig. 4b, c).

Effect of quercetin on the up-regulations of TLR-2, TLR-3 and TLR-9 protein levels by HSV-1

Host cell infection by HSV-1 is mediated by TLRs, especially TLR2, TLR3, and TLR9. Therefore, we explored whether inhibition HSV-1-induced NF-κB or IRF-3 up-regulation by quercetin is associated with Toll like receptors. HSV-1 infection increased the protein levels of TLR2, TLR3, and TLR9 in Raw 264.7 cells (Fig. 4a). Surprisingly, quercetin significantly and dose-dependently suppressed TLR-3 levels (Fig. 5a, b), but not TLR-2 and TLR-9 levels (Fig. 5a), suggesting that quercetin specifically targets TLR-3 to inhibit NF-kB and IRF-3 activation by HSV-1.

Effect of quercetin on HSV-1-induced TNF-α protein production

NF-κB-induced inflammation leads to the secretions of pro-inflammatory cytokines, including TNF-α. Thus, we examined whether quercetin inhibits TNF-α protein production in HSV-1 infected Raw 264.7 cells. Cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (10, 20, 30 μg/ml) for 24 h and ELISA was used to quantify TNF-α levels (pg/ml) in culture supernatants. HSV-1 infection alone resulted in a TNF-α concentration of ~3500 pg/ml (Fig. 6), but quercetin (30 μg/

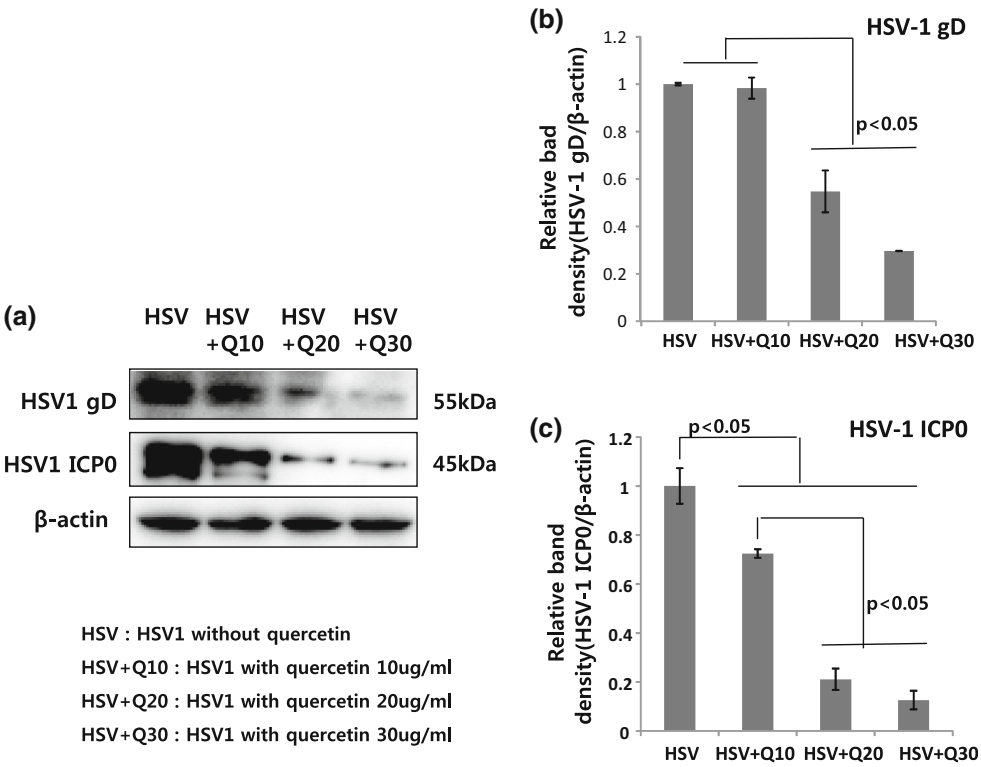

Fig. 2 Effect of quercetin on HSV-1 gD and ICP0 protein levels. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) for 24 h. Western blot analysis was performed for HSV-1 gD and ICP0 proteins. β-Actin was used as the loading control. **a** Expressions of HSV-1 gD and ICP0 proteins. **b** Relative band intensity of HSV-1 gD compared to the loading control. **c** Relative band intensity of HSV-1 gD compared to the loading control

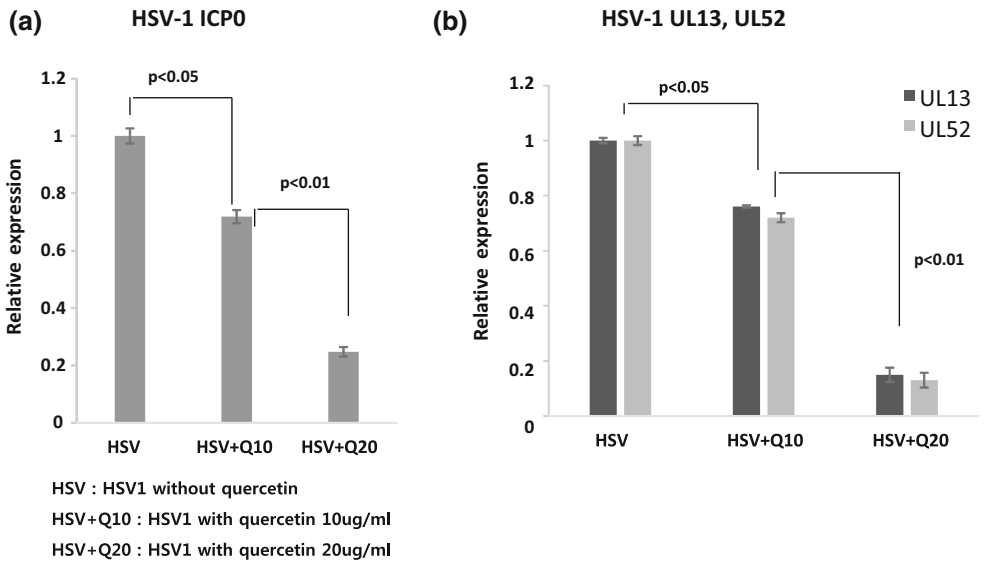

Fig. 3 Effect of quercetin on the gene expressions of HSV-1 ICP0, UL13 and UL52. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20 μg/ml) for 24 h and then total RNA was extracted. Real time qPCR was performed to determine viral ICP0, UL13 and UL52 expressions. **a** Relative gene expression of HSV-1 ICP0. **b** Relative gene expressions of HSV-1 UL13 and UL52

ml) pretreatment almost completely blocked TNF-α secretion. These observations indicate that quercetin mediates anti-inflammatory response by inhibiting TNF-α production.

Discussion

In this study, we investigated the molecular mechanism responsible for the anti-HSV-1 effect of quercetin in

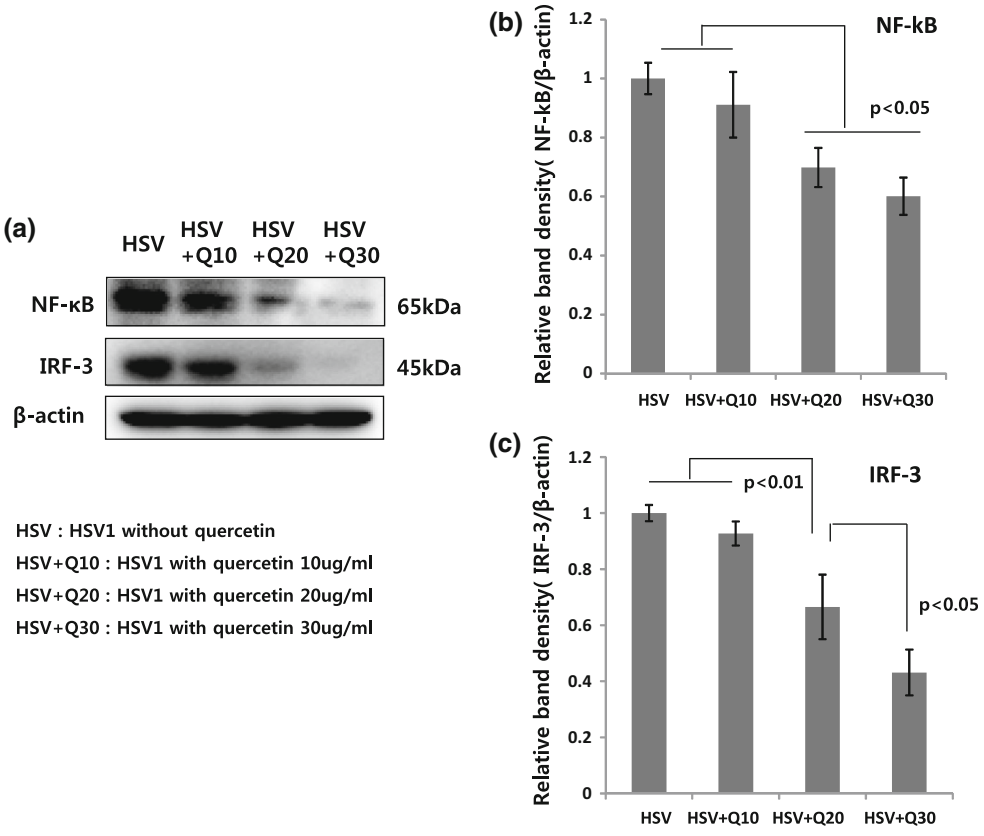

Fig. 4 Effect of quercetin on HSV-1-induced NF-κB and IRF-3 protein levels. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) for 24 h. Western blot analysis was performed for NF-κB and IRF-3. β-Actin was used as the loading control. **a** NF-κB and IRF-3 protein expressions. **b** Relative band intensity of NF-κB compared to the loading control. **c** Relative band intensity of IRF-3 compared to the loading control

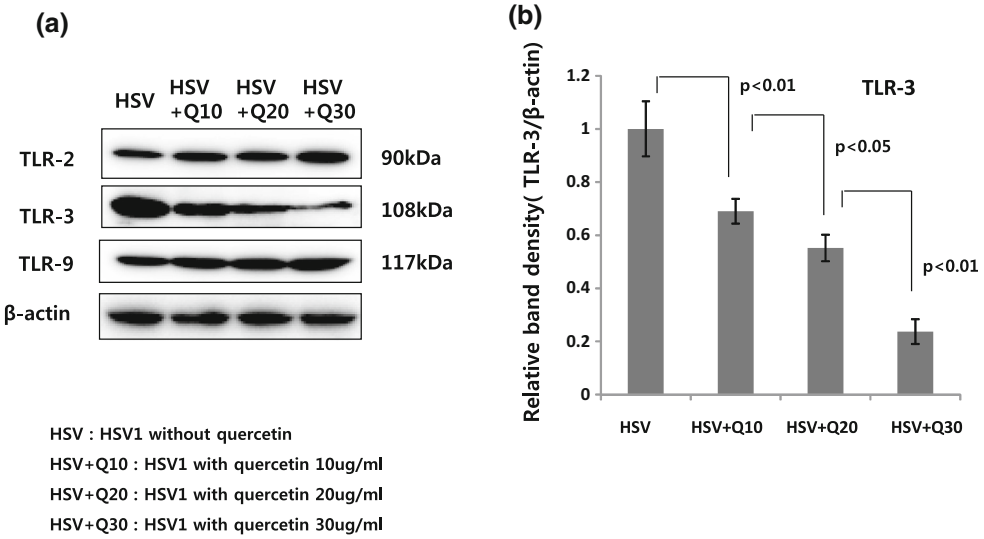

Fig. 5 Effect of quercetin on the HSV-1-induced TLR-2, TLR-3 and TLR-9 protein levels. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) for 24 h. Western blot analysis was performed for TLR-2, TLR-3 and TLR-9. β-actin was used as the loading control. **a** TLR-2, TLR-3 and TLR-9 protein expressions. **b** Relative band intensity of TLR-3 compared to the loading control

Raw264.7 cells. We found that quercetin suppressed the activations of IRF-3 and NF-κB induced by HSV-1 infection in a TLR3-dependent manner, and this resulted in reduced TNF-α production in Raw 264.7 cells.

Many studies have reported that flavonoid extracts containing quercetin have an anti-herpetic effect in different cells (Chen et al. 2011; Cho et al. 2015). Hung et al. showed that pure quercetin exerted an inhibitory effect on HSV-1 infection in Vero cells, which are highly susceptible to HSV-1 infection (Hung et al. 2015). In the present study, we used Raw 264.7 cells (a mouse macrophage cell-line) to explore the anti-HSV-1 effect of quercetin. Quercetin was observed to strongly inhibit the infectivity of HSV-1 in Raw 264.7 cells without having any significant cytotoxic effects in the dosage range used.

A number of HSV viral proteins are known to be essential for successful viral entry. Initially, viral glycoprotein B (gB), gC, and gD attach to receptors on the

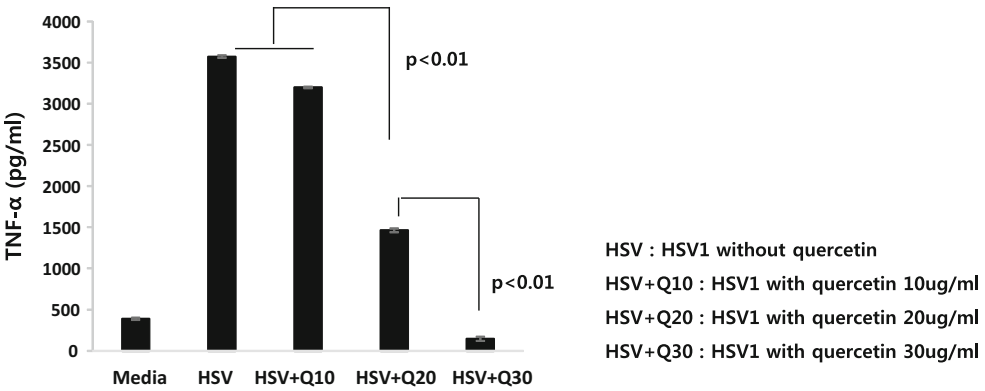

Fig. 6 Effect of quercetin on HSV-1-induced TNF-α production. Raw 264.7 cells were infected with HSV-1 at 0.1 MOI in the presence or absence of quercetin (0, 10, 20, 30 μg/ml) for 24 h, and TNF-α concentrations (pg/ml) in culture supernatants were determined by ELISA

surfaces of host cells, and then the viral envelope fuses with host plasma membrane (Spear 2004). After viral entry, HSV viral proteins, encoded by genes called immediate-early (IE), early (E), and late (L) genes, are sequentially produced. IE genes such as ICP0, ICP4, and ICP27 are the first to be expressed in the HSV-1 replication cycle (Knopf 2000; Amici et al. 2006; Moerdyk-Schauwecker et al. 2009). Of note, modulating the expressions of IE genes could contribute to control of the HSV infection cycle. Therefore, we examined whether HSV-1 gD and ICP0 protein levels in Raw 264.7 cells were affected by quercetin treatment. Hung et al. found that quercetin specifically blocked viral entry, which is directly and inversely associated with HSV-1 gD protein levels in Vero cells (Hung et al. 2015). As shown in Fig. 2, quercetin actually suppressed the expressions of HSV-1 gD and of ICP0 proteins, which suggests that quercetin affects both viral entry and viral replication, respectively. Furthermore, the inhibitory effect of quercetin on HSV-1 replication was found to be related to the down-regulation of replication-associated viral genes, such as, ICP0, UL13, and UL52 (Fig. 3).

Few studies have reported that quercetin rich extracts or pure quercetin suppressed HSV-1-induced NF-κB expression (Chen et al. 2011; Hung et al. 2015). The present study confirms the inhibitory effect of quercetin on HSV-1-induced NF-κB activation. Furthermore, it presents for the first time, quercetin also suppresses HSV-1-induced IRF-3 expression (Fig. 4). NF-κB and IRF-3 are crucial host transcriptional regulators, which can be induced by viral infections and the activations of these transcriptional factors can initiate host inflammatory processes. However, NF-κB is the final signaling factor in the MyD88-dependent pathway, and IRF-3 is the final transcriptional factor in TRIF-dependent pathways (Brasier 2006). Therefore, our results suggest that quercetin modulates the TRIF-dependent pathway in addition to the MyD88-dependent signaling pathway. Both MyD88-dependent and TRIF-dependent signaling can be started by TLRs on host cell surfaces. HSV-1 infection is primarily known to induce the activations of TLR-2, TLR-3 and TLR-9. However, whether quercetin negatively affects the activity of these TLRs or whether the inhibitory effect of quercetin on HSV-1-induced NF-κB is associated with the expression of these TLRs is not known. We found that quercetin could not modulate HSV-1-induced TLR-2 or TLR-9 activation, but could specifically attenuate the activation of TLR-3 (Fig. 5). Accordingly, our data suggest that quercetin can target HSV-1-induced TLR-3 to suppress the activations of NF-κB and IRF-3 in Raw264.7 cells. Finally, we confirmed that quercetin decreased TNF-α production, a representative inflammatory cytokine induced by HSV-1 infection, which indicates that the anti-inflammatory effect of quercetin is mediated by the suppression of HSV-1-induced NF-κB and IRF-3 expressions in TLR-3 dependent way.

In summary, we report a new anti-HSV mechanism of quercetin by which quercetin targets viral entry and viral replication. In addition, we identify the inhibition of TLR activation as a novel target to provide anti-inflammatory activity in HSV-1 infection.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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