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Kaempferol inhibited bovine herpesvirus 1 replication and LPS-induced inflammatory response

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Summary. – Plant-derived flavonoids contain large amount of compounds with pharmacological effects. In this study, we showed the compound Kaempferol to have robust antiviral activity against bovine herpesvirus 1 (BoHV-1) replication *in vitro*. Kaempferol at a concentration of 100 μmol/l completely inhibited viral replication in MDBK cells. It mainly affects the viral replication at the post-entry stages. The inhibition of Akt signaling is a potential mechanism underlying the antiviral effect of Kaempferol. In addition, at a concentration of 25 and 50 μmol/l Kaempferol could significantly reduce the expression of inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8) and macrophage inflammatory protein 1 alpha (MIP-1α) in human promonocytic U937 cells-derived macrophages (dU937) in response to lipopolysaccharides (LPS) stimulation. Overall, our results indicated that Kaempferol provides a potent protection against BoHV-1 infection and LPS-induced inflammatory response.

Keywords: BoHV-1; inflammation; Kaempferol; cytokine

Introduction

Bovine herpesvirus 1 (BoHV-1) belongs to the *Alphaherpesvirinae* subfamily (Tikoo *et al*., 1995). BoHV-1 acute infection usually results in inflammatory diseases in the upper respiratory tract, nasal cavity and ocular cavity of the cattle (Jones and Chowdhury, 2007; Jones, 2009). BoHV-1 infection suppresses the immune response, resulting in life-threatening bacterial pneumonia, referred to as bovine respiratory disease complex (BRDC) (Muylkens *et al*., 2007). BoHV-1 infections and the virus-induced BRDC inflict a great economic burden to the cattle industry worldwide, which costs the US cattle industry approximately 3 billion dollars annually (Jones and Chowdhury, 2007).

Generally, excessive expression of pro-inflammatory cytokines would exacerbate the tissue damage in the context of numerous microbial infections, e.g. the cytokine storm resulting from influenza virus H1N1 infection is a key factor for severe pneumonia (La Gruta *et al*., 2007). It has been demonstrated that the inflammatory cytokines such as interleukin 1 beta (IL-1β), TNF-α and IL-8 promote the development of BRDC (Bielefeldt Ohmann *et al*., 1991; Malazdrewich *et al*., 2001). Overproduction of inflammatory cytokines such as IL-1β and TNF-α in serum following BoHV-1 infection *in vivo* has been demonstrated (Muylkens *et al*., 2007; Risalde *et al*., 2011), which was confirmed by the report that BoHV-1 infection rapidly increases the transcription of IL-1β, IL-8 and TNF-α in the primary cell cultures of bovine bronchial epithelial cells (Rivera-Rivas *et al*., 2009). Thus, targeting

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Abbreviations: BoHV = bovine herpes virus; dU937 = U937 cells-derived macrophages; hpi = hours post infection; LPS = lipopolysaccharides; MIP-1α = macrophage inflammatory protein 1 alpha; MOI = multiplicity of infection; PMA = phorbol-12-myristate-13-acetate; PAA = phosphonoacetic acid; ROS = reactive oxidative species; TNF-α = tumor necrosis factor alpha; IL-8 = interleukin 8

both viral replication and inflammatory response represents a viable approach for the control of BoHV-1 infection.

We have recently reported that BoHV-1 infection enhanced the production of cellular reactive oxidative species (ROS), which mediate mitochondrial damage, to facilitate viral infection (Zhu *et al.*, 2016). Importantly, the inflammatory mediator ROS could also stimulate LPS-induced inflammatory response (Wang *et al.*, 2017). Increasing evidence suggests that Kaempferol has strong capacity to protect against oxidative stress, e.g. it protects normal lung and liver cells from H_2O_2-induced cytotoxicity, ROS formation and mitochondrial membrane potential loss (Kumar *et al.*, 2016). Therefore it is highly possible that Kaempferol may inhibit both BoHV-1 infection and LPS-induced inflammatory response.

In this study, we report that Kaempferol strongly suppresses BoHV-1 productive infection in cell culture, LPS-induced expression of pro-inflammatory cytokine TNF-α, and chemokines IL-8 and MIP-1α in macrophage-like dU937 cells.

Materials and Methods

Virus and cell cultures. MDBK cells were maintained in DMEM (Gibco, CA, USA) supplemented with 10% horse serum (HyClone, Logan, USA), and routinely passaged whenever they became confluent. BoHV-1 of Colorado1 strain was propagated in MDBK cells, aliquoted, titrated and stored at -70°C until use. Human promonocytic cell line U937 cells (purchased from Chinese model culture preservation center, Shanghai, China) were cultured in RPMI 1640 medium (Gibco, CA, USA) containing 10% FBS (HyClone, Logan, USA). U937 cells were *in vitro* differentiated into macrophages dU937 by induction with PMA (Sigma, MO, USA) at a concentration of 100 nmol/l for 48 h.

Reagents and antibodies. Lipopolysaccharides (LPS) (Cat# L2630), Kaempferol (Cat# K0133), phorbol-12- myristate-13-acetate (PMA) (Cat# P8139) and phosphonoacetic acid (PAA) (Cat# 284270) were obtained from Sigma-Aldrich (St. Louis, MO). They were resolved with DMSO and stored at -70°C. Antibodies for phospho-Akt (Ser473) (#9271) and total Akt (#9272), β-Actin (#4970), and horseradish peroxidase (HRP)-conjugated IgG for secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Antiviral activities assay. MDBK cells of 70–80% confluent in 24-well plates were pretreated with Kaempferol at indicated concentrations for one hour, and infected with BoHV-1 at an MOI of 1 for one hour in the presence of Kaempferol. After washing three times with phosphate-buffered saline (PBS, pH 7.4), fresh medium containing Kaempferol was replaced and cultured for 24 h. The virus yield was determined in MDBK cells with results expressed as $TCID_{50}$/ml. The compound PAA is a known anti-herpesvirus compound (Becker *et al.*, 1977). As a positive control, the antiviral effect of PAA (100 μmol/l) on BoHV-1 infection in MDBK cells was evaluated in parallel with Kaempferol (μmol/l).

Time-of-addition assay. MDBK cells of 70–80% confluent in 24-well plates were infected with BoHV-1 at MOI of 1. Kaempferol at a concentration of 100 μmol/l was added to cells at the indicated time point during virus infection. At 24 hours post infection (hpi) the virus yields were titrated in MDBK cells with results expressed as $TCID_{50}$/ml. The diagram in Fig. 2a shows four different experimental conditions.

Cell lysate preparation and Western blotting analysis. MDBK cells cultured in 60-mm dishes were mock infected with medium or infected with BoHV-1 at MOI of 10 along with or without Kaempferol. At 30 min post infection, the cells were washed and lysed with lysis buffer (1% Triton X-100, 50 mmol/l sodium chloride, 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mmol/l sodium fluoride, 20 mmol/l sodium pyrophosphate, 1 mmol/l phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1 mmol/l benzamidine, and 1 mmol/l sodium orthovanadate in 20 mmol/l Tris-HCl, pH 8.0). The cell lysates were then subjected to Western blotting analysis.

Detection of cytokines with ELISA. dU937 cells in 6-well plates were mock-treated with PBS or treated with LPS (1 μg/ml) in the absence or presence of Kaempferol at indicated concentrations for 24 h. The medium was collected and subjected to the detection of cytokines IL-8, TNF-α and MIP-1α with commercial ELISA kits from Boster Biological Technology (Hubei, China).

Statistical analysis. These studies were repeated 3 times and an asterisk denotes significant differences between vehicle and Kaempferol (* $P < 0.05$ and ** $P < 0.01$) as determined by the Student's *t*-test.

Results

The antiviral effect of Kaempferol on BoHV-1 infection

To assess the antiviral effect of Kaempferol on BoHV-1 replication, a concentration of 100 μmol/l that has no cytotoxicity to MDBK cells was selected by MTT assay (data not shown). MDBK cells were first pretreated with DMSO or Kaempferol at indicated concentrations of 25, 50 and 100 μmol/l, then infected with BoHV-1 (MOI = 1). The cells were treated with Kaempferol through the virus infection. At 24 hpi the virus yield was determined. As expected, Kaempferol significantly inhibited viral replication in a dose-dependent manner. Surprisingly, at the concentration of 100 μmol/l, Kaempferol completely suppressed the virus replication, while at the concentration of 25 and 50 μmol/l it reduced the viral titer by ~ 1.1 log and 3.6 log, respectively (Fig. 1a). PAA is a specific inhibitor targeting the viral DNA polymerase (Becker *et al.*, 1977). The antiviral effect of Kaempferol on BoHV-1 infection was further analyzed through comparison to PAA. Kaempferol demonstrated much stronger inhibitory effect on viral replication relative to PAA. PAA at a concentration of 100 μmol/l re-

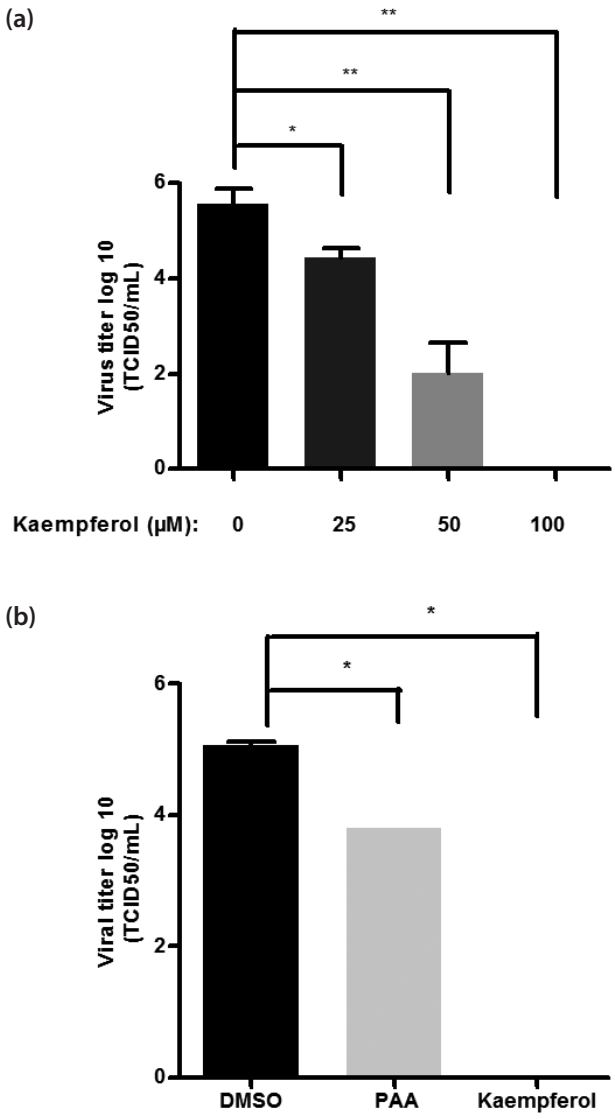

Fig. 1

Kaempferol inhibited BoHV-1 replication in MDBK cells

(a) MDBK cells in 24-well plates were pretreated with DMSO control or Kaempferol at indicated concentrations for 1 h, infected with BoHV-1 at MOI 1 for 1 h in the presence of Kaempferol. At 24 h after infection, cells together with medium were collected for virus titration. **(b)** MDBK cells in 24-well plates were pretreated with DMSO control or Kaempferol or PAA at indicated concentrations for 1 h, then infected with BoHV-1 at MOI 1 for 1 h in the presence of Kaempferol or PAA. At 24 h after infection, cells together with medium were collected for virus titration. Data presented are mean ± SD of three independent experiments. Significance was assessed by Student's *t* test (*, P <0.05 and **, P <0.01).

duced the virus titer by ~1.3 log, while Kaempferol completely inhibited virus replication (Fig. 1b), which also validated the antiviral effect of Kaempferol.

To clarify which step(s) of BoHV-1 replication were affected by Kaempferol, a time-of-addition assay was performed.

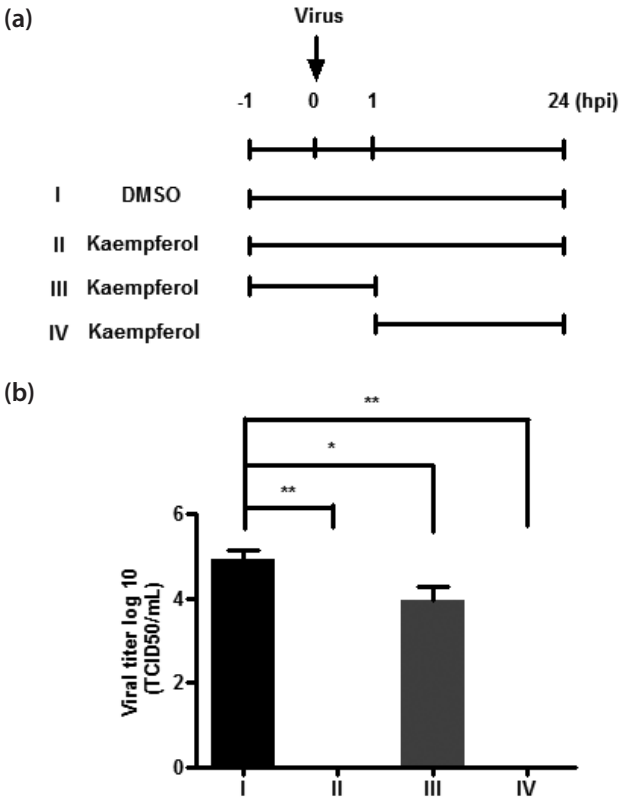

Fig. 2

(a) Diagram showing four different experimental conditions. I, DMSO treatment from -1 to 24 hpi. II, Kaempferol treatment from -1 to 24 hpi. III, Kaempferol treatment from -1 to 1 hpi. IV, Kaempferol treatment from 1 to 24 hpi. (b) Viral production of BoHV-1 under experimental conditions I to IV. Data presented are mean ± SD of three independent experiments. Significance was assessed by Student's *t* test (*, P <0.05 and **, P <0.01).

MDBK cells in 24-well plates were treated with Kaempferol at a concentration of 100 μmol/l in different manners as shown in Fig. 2a. The treatment of cells with Kaempferol at the post-entry stages (III) still completely inhibited the virus replication (Fig. 2b), suggesting that Kaempferol may interrupt BoHV-1 infection mainly at the post-entry stage(s). When cells were exposed to Kaempferol at both virus binding and entry stages (II), the virus replication was still significantly inhibited, with ~1 log reduction compared to the control. This reduction may have been caused by the residual intracellular Kaempferol that would affect the virus replication at the post-entry stages.

Kaempferol inhibited Akt signaling in response to BoHV-1 infection

We have previously identified that BoHV-1 infection stimulated Akt signaling for efficient replication, and this

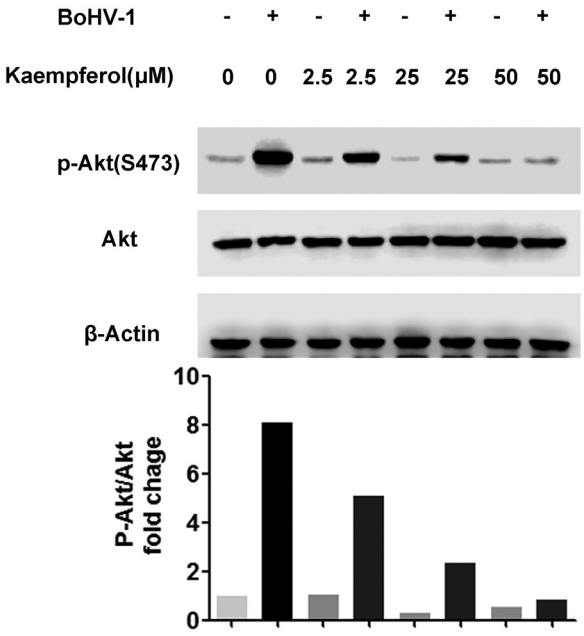

Fig. 3

Kaempferol interfered with Akt signaling following BoHV-1 infection in MDBK cells

MDBK cells were treated with or without Kaempferol at indicated concentrations for 1 h, then infected with BoHV-1 at MOI of 10 for 0.5 h, along with the treatment of Kaempferol at corresponding concentrations. The cell lysates were prepared for Western blotting analysis by the detection of phospho-Akt, Akt and β-Actin. Quantitative analysis was performed with Image J software. These results are representative of three independent experiments.

signaling mainly affects the virus post-entry stages (Zhu *et al*., 2011). Therefore, the effect of Kaempferol on Akt signaling stimulated by BoHV-1 infection was evaluated. MDBK cells were infected with BoHV-1 (MOI = 10) along with the treatment with Kaempferol at concentrations of 50, 25 and 2.5 μmol/l. As a result, Kaempferol diminished the activation of Akt signaling in response to BoHV-1 infection in a dose-dependent manner (Fig. 3), suggesting that the interruption of Akt signaling is a potential mechanism for the antiviral effect of Kaempferol against BoHV-1 infection.

Kaempferol inhibited LPS-induced expression of proinflammatory cytokine TNF-α and chemokines IL-8 and MIP-1α

Monocytes/macrophages are one of the main sources that produce inflammatory cytokines and chemokines. LPS is a canonical stimulator for the induction of inflammatory cytokines. Here, the effect of Kaempferol on LPS-induced expression of pro-inflammatory cytokine TNF-α, and the chemokines IL-8 and MIP-1α in dU937 cells was evaluated

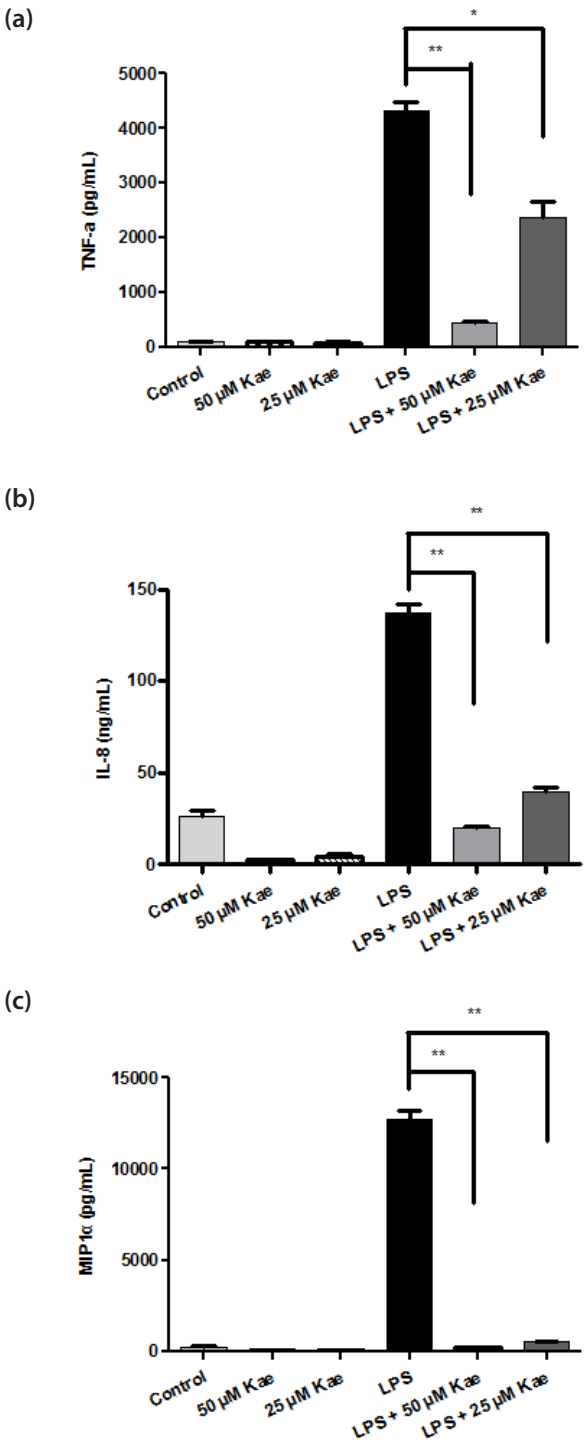

Fig. 4

Kaempferol inhibited the expression of (a) TNF-α, (b) IL-8 and (c) MIP-1α in macrophage-like dU937 cells

dU937 cells were mock stimulated with DMSO or stimulated with LPS (1 μg/ml) in the presence of Kaempferol at various concentrations. At 24 h post stimulation, the culture medium was collected for the detection of TNF-α, IL-8 and MIP-1α with ELISA kits. Data presented are mean ± SD of three independent experiments. Significance was assessed with Student's *t* test (*, $P < 0.05$ and **, $P < 0.01$).

with commercial ELISA kit. dU937 cells were stimulated with LPS (1 μg/ml) in the presence of DMSO control or Kaempferol at indicated concentrations, respectively. The medium was collected for subsequent detection of indicated inflammatory cytokines and chemokines. The LPS-induced expression of TNF-α, IL-8 and MIP-1α was inhibited by Kaempferol in a dose-dependent manner (Fig. 4). LPS induced the expression of TNF-α to a concentration of 4325.73 pg/ml, while the treatment of cells with Kaempferol at a concentration of 25 and 50 μmol/l reduced TNF-α expression to a concentration of 2358.91 and 443.45 pg/ml, respectively (Fig. 4a). LPS induced the IL-8 expression to a concentration of 137.47 ng/ml, which was reduced to a concentration of 19.72 and 39.50 ng/ml by 50 and 25 μmol/l Kaempferol, respectively (Fig. 4b). LPS induced MIP-1α expression to a concentration of 12732.4 pg/ml, which was reduced by Kaempferol at a concentration of 25 and 50 μmol/l to a concentration of 220.37 and 536.69 ng/ml, respectively (Fig. 4c). These data suggested that Kaempferol has strong capacity to lower the production of pro-inflammatory cytokine TNF-α and chemokines IL-8 and MIP-1α in macrophages like dU937 cells.

Discussion

Plant-derived flavonoids and dietary isoflavones are a large group of natural phenylchromones found in fruits, vegetables, tea, soy foods, and herbs. Many flavonoids have been shown to possess multiple pharmacological effects such as antioxidative activity, anti-inflammatory and anticancer activities, as well as antiviral activities (Orhan *et al*., 2010; Kumar and Pandey, 2013). The flavonoid Kaempferol has been demonstrated to have antiviral activity against influenza viruses (H1N1 and H9N2), hepatitis B virus, Japanese encephalitis virus, enterovirus 71, HIV-1 and chikungunya virus *in vitro* (Li *et al*., 2008; Jeong *et al*., 2009a; Tsai *et al*., 2011; Zhang *et al*., 2012; Behbahani *et al*., 2014; Lani *et al*., 2015). But whether Kaempferol has anti-herpesvirus effect was unknown until now. In this study, we identified for the first time that Kaempferol had strong antiviral effect on BoHV-1 (Fig. 1). Since mentioned viruses belong to different families, it is highly possible that Kaempferol may have broad spectrum of antiviral effects. In view that Kaempferol showed a stronger antiviral activity relative to the known anti-herpesvirus drug PAA, this study may have important consequence to broaden the possiblity of getting an effective antiviral for herpesvirus infection.

Multiple evidence has indicated that Kaempferol could interrupt Akt signaling in multiple cell cultures, e.g. Kaempferol causes G2/M arrest in human hepatic cancer cells via induction of autophagy partially through Akt-dependent mechanism, and Kaempferol induces cell death in glioma cells partially through the inhibition of Akt signaling (Jeong *et al*., 2009b; Huang *et al*., 2013). These reports corroborated our result that Kaempferol inhibited Akt signaling in bovine kidney cells stimulated by BoHV-1 infection (Fig. 3). Thus, the damping of Akt signaling is a possible mechanism of the antiviral effect of Kaempferol.

Kaempferol could attenuate LPS-induced production of NO, PGE, TNF-α, IL-1β and ROS in BV2 microglial cells (Park *et al*., 2011). Here, our results indicated that Kaempferol significantly suppressed the production of TNF-α, IL-8 and MIP-1α in macrophage-like dU937 cells, suggesting that Kaempferol also affects the inflammatory response in monocyte/macrophages. Thus Kaempferol may have potential anti-inflammatory effect mediated by multiple cell types.

In summary, for the first time we revealed that Kaempferol has both antiviral effect against BoHV-1 infection and anti-inflammatory effects mediated by macrophages.

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